(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
3 January 2003 (03.01.2003)

PCT

(10) International Publication Number
WO 03/000232 A2

(51) International Patent Classification[7]: A61K 9/127, 39/39

(21) International Application Number: PCT/IL02/00507

(22) International Filing Date: 25 June 2002 (25.06.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/300,072 25 June 2001 (25.06.2001) US
60/339,785 17 December 2001 (17.12.2001) US

(71) Applicants *(for all designated States except US)*: **YISSUM RESEARCH DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM** [IL/IL]; 46 Jabotinsky Street, 92182 Jerusalem (IL). **THE REGENTS OF THE UNIVERSITY OF CALIFORNIA** [US/US]; 1111 Franklin Street, 12th Floor, Oakland, CA 94607-5200 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **BARENHOLZ, Yechezkel** [IL/IL]; 18 Nave Shaanan Street, 93707 Jerusalem (IL). **KEDAR, Eliezer** [IL/IL]; 3 Eli Cohen Street, 92347 Jerusalem (IL). **LOURIA-HAYON, Yigal** [IL/IL]; Beth Louria, Hamat Hamaiem Street, 30300 Atlit (IL). **JOSEPH, Aviva** [IL/IL]; 26 Yitzhak Tunik Street, Pizgat Zeev, 97795 Jerusalem (IL). **RAZ, Eyal** [IL/US]; 13658 Mango Drive, Del Mar, CA 92014 (US). **TAKABAYASHI, Kenji** [JP/US]; 8959 Gainsborough Ave, San Diego, CA 92129 (US).

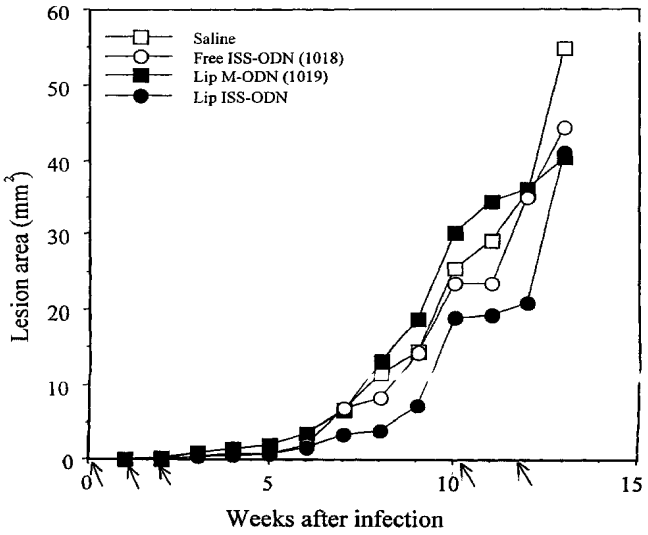
(74) Agent: **REINHOLD COHN AND PARTNERS**; P.O.Box 4060, 61040 Tel Aviv (IL).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),

[Continued on next page]

(54) Title: A METHOD FOR PREPARATION OF VESICLES LOADED WITH IMMUNOSTIMULATORY OLIGODEOXYNUCLEOTIDES AND DIFFERENT USES THEREOF



(57) Abstract: The present invention is based on the finding of a simple and fast method for an effective entrapment of active ISS-ODN in liposomes, with more than 60% loading). The method is based on drying a suspension of amphipathic material and then hydrating it with an aqueous solution containing the ISS-ODN thereby entrapping it in liposomes formed from the lipid. Thus, the present invention concerns an efficient method for loading, and retaining, ISS-ODN in liposomal vesicles.Animals treated with liposomal ISS-ODN of the invention are shown to demonstrate a slower tempo of disease development as compared to free ISS-ODN or to liposomes loaded with mutant ISS-ODN (lip M-ODN).

WO 03/000232 A2

Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

A METHOD FOR PREPARATION OF VESICLES LOADED WITH IMMUNOSTIMULATORY OLIGODEOXYNUCLEOTIDES AND DIFFERENT USES THEREOF

FIELD OF THE INVENTION

This invention generally relates to liposomal formulations and in particular to a method for the preparation of liposome vesicles loaded with immunostimulatory oligodeoxynucleotides (ISS-ODN) and to the different uses of its products.

PRIOR ART

The following is a list of prior art which is considered to be pertinent for describing the state of the art in the field of the invention.

(1) Lichtenberg D., and Barenholz Y. in Methods of Biochemical Analysis (Glick D., Ed.) Wiley NY pp. 337-462, 1988;

(2) Barenholz Y., and Crommelin D.J.A., in Encyclopeida of Pharmaceutical Technology (Swabrick J and Boylan J.C. Eds.) Vol. 9, Marcel Dekker NY pp. 1-39 (1994);

(3) US Patent No. 6,156,337;

(4) US Patent No. 6,066,331;

(5) C. Kirby and G. Gregoriadis Bio/Technology, November 1984, pages 979-984;

(6) Van Uden J., and Raz, E. in *Springer Semin. Immunopathol.* **22:** 1-9 (2000);

(7) McCluskie, M.J., *et al. Vaccine*, **19:**2657-2660 (2001);

(8) Horner, A.A., *et al. Immunol Rev.* **179**,102-118 (2001);

(9) Klinman, D.M., *et al. Springer Semin. Immunopathol 22*:173-183 (2000);

(10) Wagner, H., *et al. Springer Semin. Immunopathol.* **22**:167-171 (2000);

(11) Diminsky, *et al., Vaccine,* **15**:637-647 (1997);

(12) Alving, CR. (1997) in *New generation vaccines, 2nd ed.* (Levine, M.M., Woodrow, G.C., Kaper, J.B., and Cobon, G.S., eds.), Marcel Dekker, New York, pp. 207-213;

(13) Kedar, E. and Barenholz, Y. (1998) in *The biotherapy of cancers: from immunotherapy to gene therapy* (Chouaib S, ed.), *INSERM,* Paris, *pp.* 333-362.

BACKGROUND OF THE INVENTION

Several attempts have been made to use lipid vesicles formed by natural or synthetic phospholipids as vehicles for the administration of effective substances. Proposed clinical uses have included vaccine adjuvanticity, gene transfer and diagnostic imaging, but the major effort has been in the development of liposomes as targetable drug carriers in the treatment of malignancy.

Amphotericin B, an effective but toxic antifungal, was the first liposomally formulated agent to be licensed for parenteral use.

Antitumor agents like adriamycin (doxorubicin) have also been incorporated into liposomes, as well as vaccines, adjuvants and biological response modifiers like cytokines and others.

Liposomes are also utilized as vehicles in the field of gene transfer [Kastel P.L, and Greenstein R.J., *Biotechnol. Annu. Rev.* **5**:197-220 (2000)]. In another application, liposomes were used for the delivery of therapeutic proteins. N. Sakuragawa *et al.* [Thrombosis Research **38**:681-685, (1985); Clinical Hematology 29(5):655-661 (1988)]report that liposomes containing factor VIII have been prepared for oral administration to patients suffering from von Willebrand's disease.

The encapsulation of factor VIII was carried out by dissolving the protein factor VIII concentrates in an aprotinin containing solution and transferred into lecithin coated flasks. After drying the flasks by rotation for 30 min under negative pressure liposomes were formed which entrapped factor VIII concentrates. The liposome suspension was centrifuged yielding 40% of factor VIII entrapped in liposomes.

Another method for entrapment of drugs in liposomes is based on a procedure referred to as the dehydration-re-hydration procedure. This is described by C. Kirby and G. Gregoriadis [Bio/Technology, November 1984, pages 979-984]. In this preparation the entrapment was increased by using additional lipid and the use of cholesterol is described as having positive influence of the drug entrapment.

Yet another method for loading vesicles with biological substances is described in US Patent Nos. 6,066,331 and 6,156,337. According to the method described therein, liposomes loaded with biological structures, biopolymers and/or oligomers, are obtained by co-drying a fraction of an amphipathic material in an organic solvent and a fraction of the biological structure(s), biopolymers and/or oligomers, from an aqueous medium.

The present invention aims for the providence of a novel method for efficient encapsulation (≥60%) of oligomers, particularly those being pharmaceutically active, into lipid membrane vesicles.

A group of oligomers of particular interest according to the present invention are oligonucleotides and especially, the immunostimulatory oligodeoxynucleotides and analogs (ISS-ODN or CpG motifs). Typically, ISS-ODN are short synthetic oligodeoxynucleotides (6-30 bases) usually containing an active 6-mer sequence that has the general structure of two 5' purines, an unmethylated CpG dinucleotide, and two 3' pyrimidines (Pu-Pu-CpG-Pyr-Pyr).

Bacterial DNA and its synthetic ISS-ODN are known to be potent stimulators of both innate immunity and specific adaptive immune responses, including direct activation of monocytes/macrophages, dendritic cells, NK cells and B cells. Further, bacterial DNA and its synthetic ISS-ODN induce the production of pro-inflammatory cytokines (e.g., IL-6, IL-12, IFNs, TNFα) and up-regulate the expression of MHC I, MHC II and co-stimulatory molecules [Van Uden J., and Raz, E. in *Springer Semin. Immunopathol.* **22**:1-9 (2000)].

In animal studies, ISS-ODNs exhibit strong Th1 and mucosal adjuvanticity to a wide range of antigens [McCluskie, M.J., *et al. Vaccine*, **19**:2657-2660 (2001)] or

allergens [Horner, A.A., *et al. Immunol Rev.* **179**:102-118 (2001)]. Furthermore, pretreatment with ISS-ODN, even without concomitant administration of the relevant antigen, was shown to afford protection (for about 2 weeks) against subsequent infection with intracellular pathogens [Klinman, D.M., *et al.* in *Springer Semin. Immunopathol* ***22***:173-183 (2000)], indicating activation of innate immunity.

The immunostimulatory activity of ISS-ODNs requires cellular uptake by endocystosis following their binding to a receptor belonging to the Toll-like receptor family, TLR9. Endosomal acidification and digestion of the ODN followed by interaction with specific protein kinases results in rapid generation of reactive oxygen intermediates, leading to activation of MAPK and NF- κB pathways and subsequent cytokine production (Chu, W., *et al. Cell* **103**:909-918 (2000)].

In mice, doses of 50-100 μg per mouse of soluble ISS-ODN, and in many cases two or more administrations were required to achieve the desired immunomodulatory effects. This relatively high dose and repeated administration, in theory, may cause adverse reactions resulting from the "cytokine storm" induced [Wagner, H., *et al. Springer Semin. Immunopathol.* **22**:167-171 (2000)].

As liposomes can effectively entrap various drugs, which are slowly released over an extended period of time *in vivo*, and can rapidly and effectively be uptaken by macrophages and dendritic cells, it is suggested that liposomes can serve as an efficient delivery system for ISS-ODN based vaccines [Alving, CR. (1997) in *New generation vaccines*, *2nd ed.* (Levine, M.M., Woodrow, G.C., Kaper, J.B., and Cobon, G.S., eds.), Marcel Dekker, New York, pp. 207-213; and Kedar, E. and Barenholz, Y. (1998) in *The biotherapy of cancers: from immunotherapy to gene therapy* (Chouaib S, ed.), *INSERM,* Paris, *pp.* 333-362].

SUMMARY OF THE INVENTION

The present invention is based on the surprising finding that step-wise hydration of lipids, *a priori* freeze dried, with an aqueous solution containing ISS-ODN to be encapsulated in said liposomes, results in a very effective encapsulation of the ISS-ODN as compared to hitherto known encapsulation methods.

Thus, according to a first of its aspects, the present invention provides a method for loading ISS-ODN in liposomes, the method comprising:

(1) solubilizing at least one liposome-forming lipid in a solvent and drying the same to effect a dry liposome-forming lipid or a dry mixture of such lipids;

(2) providing an aqueous solution of ISS-ODN or of a mixture of ISS-ODNs;

(3) hydrating said liposome-forming lipid or said liposome-forming lipids with said ISS-ODN to yield liposomes loaded with said ISS-ODN.

The term "***liposome***" as used includes all spheres or vesicles of amphipathic substance which may spontaneously or non-spontaneously vesiculate, for example phospholipids, where at least one acyl group is replaced by a complex phosphoric acid ester.

The term "***loading***" means any kind of interaction of the oligomeric substances to be loaded with the liposomes, for example, an interaction such as encapsulation, adhesion, entrapment (to the inner or outer wall of the vesicle) or embedding in the liposome membrane, with or without extrusion of the ISS-ODN.

Also as used herein, the term "***liposome-forming lipid***" denotes any physiologically acceptable amphipathic substance that contains groups with characteristically different properties, e.g. both hydrophilic and hydrophobic properties or a mixture of such molecules, and which upon dispersion thereof in an aqueous medium, form vesicles. As will be further elaborated hereinafter, this term refers to a single amphipathic substance or to a mixture of such substances. The amphipathic substance includes, *inter alia*, phospholipids, sphingolipids, glycolipids, such as

cerebrosides and gangliosides; PEGylated lipids and sterols, such as cholesterol and others.

The terms "***dry***" or "***drying***" refer to any manner of drying the liposome-forming lipids, which results in the formation of a dry lipid cake. According to one preferred embodiment, drying is achieved by freeze drying, also referred to as lyophilizing. Alternatively, drying may be achieved by spray drying.

The term "***solubilizing***" which is used herein interchangeably with the term "***dissolving***" or "***dispersing***" may be achieved by a single use of the bulk aqueous medium with which said solubilization is achieved. However, this term preferably refers to step-wise addition of two or more aliquots of the said medium solubilizing the solute.

The method of the invention will at times be referred to in the following description by the term "***post-encapsulation***" ("POST"), according to which dry lipids are hydrated with an aqueous solution containing the ISS-ODN. This is as opposed to Co-encapsulation technique. "***Co-encapsulation***" ("CO") is an encapsulation method, which includes co-drying the liposome forming lipid(s) and the ISS-ODN (co-lyophilized) after which they are hydrated together with an aqueous medium.

One feature of the ***post encapsulation*** methodology disclosed herein is that it does not necessitate the freeze-drying of the ISS-ODN. As may be appreciated, there are numerous biological substances which are sensitive to lyophilization, which procedure result in deactivation of the biological substance. In addition, by the method of the present invention ISS-ODN does not need to be exposed to any organic solvent or detergent that may be destructive to its activity.

The method of the present invention enables to obtain vesicles with substantially high loading rate of ISS-ODNs (more than 60%). This feature is unique since it improves efficiency of treatment or prophylaxis with ISS-ODN loaded into the liposomes as well as it enables to reduce the dose and frequency/number of administrations required in order to achieve a desired therapeutic effect.

Further, since by the present invention, the liposome-forming lipids and the ISS-ODN are kept separately, it enables combinatorial formulations, i.e. the physician may prescribe any combination of liposome-forming(s) substance and biological agent, and upon need, the pharmacist can easily formulate the selected combination.

Further, by the present invention, the freeze-dried lipids are provided with a long shelf-life at 4°C or at room temperature, preserving their entrapment capability for over two years (data not shown), and the hydration of the lipids with the ISS-ODN solution to form the liposomes is very simple and requires only several minutes. Therefore, the liposomal formulation can be readily prepared before treatment (bedside), ensuring high pharmaceutical stability of the formulation and without leakage of the entrapped material from the liposome.

According to a second aspect, there is provided by the present invention a combination of two compositions including a first composition comprising dry liposome-forming lipids and a second composition comprising ISS-ODN, the combination is for use in the preparation of a pharmaceutical formulation comprising liposomal ISS-ODN.

The combination of the invention may be obtained in the form of a package. Accordingly, the present invention also concerns a combination of at least one composition of dry liposome-forming lipid or a dry mixture of liposome-forming lipids; and at least one composition comprising ISS-ODN; further comprising instructions for selection and use of the first and second compositions for the preparation of a therapeutic formulation, said instructions comprising hydrating said dry liposome-forming lipid(s) with an aqueous solution of said ISS-ODN to yield a pharmaceutical formulation comprising liposomes loaded with ISS-ODN; and further comprising instructions prescribing administration of said pharmaceutical formulation to a subject in need of said formulation.

The present invention also concerns pharmaceutical formulations comprising as active ingredient a therapeutically effective amount of liposomal ISS-ODN; the liposomal ISS-ODN being prepared by the method of the invention as disclosed hereinabove and below.

The pharmaceutically "***effective amount***", including also a prophylactically effective amount, for purposes herein, is determined by such considerations as are known in the art. The amount refers to that of ISS-ODN must be effective to achieve the desired therapeutic effect.

According to yet a further aspect of the invention there are provided therapeutic methods comprising administration to a subject in need an effective amount of liposomal ISS-ODN prepared according to the present invention, optionally in combination with other active agents, such as antigens. According to one embodiment, the therapeutic method comprises the prevention or treatment of a disease.

The terms ***"prevention or treatment"*** or ***"treatment"*** as used herein refer to administering of a therapeutic amount of liposomal ISS-ODN which is effective to ameliorate undesired symptoms associated with a disease, to prevent the manifestation of such symptoms before they occur, to slow down the progression of the disease, slow down the deterioration of symptoms, to enhance the onset of remission period, slow down the irreversible damage caused in the progressive chronic stage of the disease, to delay the onset of said progressive stage, to lessen the severity or cure the disease, to improve survival rate or more rapid recovery, to prevent the disease form occurring, to prime the immune response against the disease to be treated, or a combination of two or more of the above. In addition, the term ***"treatment"*** in the context used herein refers to prevention of a disease from occurring. The treatment (also preventative treatment) regimen and the specific formulations to be administered will depend on the type of disease to be treated and may be determined by various considerations known to those skilled in the art of medicine, e.g. the physicians.

Finally, the invention concerns a method for achieving a therapeutic effect, the therapeutic effect comprising stimulating an immune response of an individual, the method comprising administration to said individual an amount of liposomal ISS-ODN effective to achieve said therapeutic effect, wherein said effect is to an extent greater than that obtained by administration to the individual free ISS-ODN, the liposomal ISS-IDN being prepared by the method of the invention.

The amount administered to the individual, is a dosage of up to 2,000 mg of ISS-ODN loaded liposomal vesicles (measured by phospholipid), per kg body weight (wt).

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows the therapeutic effect of Leishmanial infection in mice by free ISS-ODN (ODN 1018, ○), liposomal M-ODN (ODN 1019, ■), or liposomal ISS-ODN, (●), Saline served as the control (□). Leishmania major promastigotes (250,000) are injected s.c. on day 0; i.m. injection of ODN (20 ug, free or liposomal) on day 1, 7, 14, 72 and 82 (↖); n=10 Balb/c mice per group.

Fig. 2 shows the therapeutic effect of Leishmanial infection by a free anti-Leishmania drug, Amphotericin B (Fungizone, ○), liposomal ISS-ODN (ODN 1018, ■), or liposomal ISS-ODN in combination with Fungizone (●); Saline served as the control (□). Leishmania major promastigotes (250,000) were injected s.c. on day 0; ODN was injected i.m. (20 ug, free or liposomal) on day 7, 14, 21, and 28 (↖); and Fungizone was injected s.c. (20 ug) on day 7, 9, 12, 15 and 18; n=10 Balb/c mice per group.

Fig. 3 shows the therapeutic effect of Leishmanial infection by free Amphotericin B derivative (AmB-AG, ●), Liposomal M-ODN (ODN 1019, ■), liposomal ISS-ODN (ODN 1018, ○), and liposomal ISS-ODN in combination with AmB-AG (△); Saline served as the control (□). Leishmania major promastigotes (250,000) were injected s.c. on day 0; ODN was injected i.m. (20 ug, free or liposomal) on day 1, 7, 14, and 21 (↖); and AmB-AG was injected s.c. (20 ug) on day 1, 3, 5, and 7; n=10 Balb/c mice per group

DETAILED DESCRIPTION OF THE INVENTION

Liposomes can be classified according to various parameters. For example, when size and number of lamellae (structural parameters) are used, four major types of liposomes are identified: multilamellar vesicles (MLV), small unilamellar vesicles (SUV), large unilamellar vesicles (LUV) and oligolamellar vesicles (OLV).

MLV form spontaneously upon hydration of dried phospholipids above their gel to liquid crystalline phase transition temperature (Tm). Their size and shape are heterogeneous and their exact structure is determined by their method of preparation [Barenholz, Y. and Crommelin, D.J.A., (1994) *ibid.*]. In general, MLV have an aqueous and lipid component separated by bilayers.

SUV are formed from MLV by sonication or by extrusion and are single bilayered (<100 nm). They are the smallest species with a high surveillance and high surface-to-volume ratio and hence have the lowest capture volume of aqueous space to weight of lipid.

The third type of liposome according to this classification includes large (> 100 nm) unilamellar vesicles (LUV) having one large aqueous compartment and a single lipid bilayer, while the fourth type of liposome includes oligolamellar vesicles (OLV), which are vesicles containing few lamellae (lipid bilayers).

Liposomes are formed from amphipathic compounds, which may spontaneously or non-spontaneously vesiculate. Such amphipathic compounds typically include triacylglycerols where at least one acyl group is replaced by a polar and/or charged moiety, e.g. phospholipids formed by a complex phosphoric acid ester. Other groups of liposome-forming lipids are the sphingolipids such as sphingomyelin (N-acyl sphingosine-phosphocholine). Examples of sphingomyelins (SPM) include (but are not limited to) egg-derived SPM, milk-derived SPM, N-palmitoyl-SPM, N-stearoyl-SPM, N-oleoyl-SPM (C18:1), N-nervacyl C (C24:1) SPM, N-lignoceryl SPM (C24:0), and their mixtures.

Any commonly known liposome-forming lipid may be suitable for use by the method of the present invention. The liposome-forming lipids can also be produced synthetically. The source of the lipid or its method of synthesis is not critical: any naturally occurring lipid, with and without modification, or a synthetic phosphatide can be used.

According to one embodiment, the lipids are phospholipids.

Examples of specific phosphatides are L-α-(distearoyl) lecithin, L-α-(diapalmitoyl) lecithin, L-α-phosphatide acid, L-α-(dilauroyl)-phosphatidic acid, L-α(dimyristoyl) phosphatidic acid, L-α(dioleoyl)phosphatidic acid, DL-α(dipalmitoyl) phosphatidic acid, L-α(distearoyl) phosphatidic acid, and the various types of L-α-phosphatidylcholines prepared from brain, liver, egg yolk, heart, soybean and the like, or synthetically, and salts thereof. Other suitable modifications include the controlled peroxidation of the fatty acyl residue cross-linkers in the phosphatidylcholines (PC) and the zwitterionic amphiphates which form micelles by themselves or when mixed with the PCs such as alkyl analogues of PC.

According to one embodiment, lecithines (also known as phosphatidylcholines (PC)) are used, which are mixtures of the diglycerides of stearic, palmitic, and oleic acids linked to the choline ester of phosphoric acid. The lecithines are found in all animals and plants such as eggs, soybeans, and animal tissues (brain, heart, and the like). These can be modified by partial or complete hydrogenation to produce partial or fully hydrogenated phospholipids (i.e. hydrogenated soy phosphatidylcholine).

The phospholipids can vary in purity and can also be hydrogenated either fully or partially. Hydrogenation reduces the level of unwanted peroxidation, and modifies and controls the gel to liquid/crystalline phase main transition temperature (T_m) which effects packing and leakage.

The liposomes can be "tailored" to the requirements of any specific reservoir including various biological fluids, which maintain their stability without aggregation or chromatographic separation, and thereby remain well dispersed and suspended in the injected fluid. The fluidity *in situ* changes due to the composition, temperature, salinity, bivalent ions and presence of proteins. The liposomes can be used with or without any other solvent or surfactant.

The liposomes may contain a combination of lipid components, as long as this combination does not induce instability, aggregation and/or chromatographic separation (demixing). This can be determined by routine experimentation, known to those in the art.

A variety of methods for producing the different types of liposomes are known and available of such methods include, for example:

1. Hydrating a thin dried film of a phospholipid with an aqueous medium followed by mechanical shaking, ultrasonic irradiation and/or extrusion of the liposome thus formed through a suitable filter;
2. Dissolving a lipid in a suitable organic solvent, mixing with an aqueous medium followed by removal of the solvent;
3. By use of a gas above its critical point (i.e., freon and other gases such as CO_2 or mixtures of CO_2 and other gaseous hydrocarbons) or
4. Preparing lipid detergent mixed micelles followed by lowering the concentration of the detergent to a level below its critical concentration at which liposomes are formed [Lichtenberg D and Barenholz Y. (1988) *ibid.*].
5. Hydrating (dispersing) with aqueous medium dry liposomes loaded with an active agent (US 66,066,331, US 6,156,337).

One obstacle when using liposomes as a drug delivery tool, are the potential destructive/inactivating effect of the loading process on the biological material to be loaded into the liposome and the efficiency of loading of the biologically effective material, and the physical stability of the liposomal formulation with storage time. For water-soluble expensive drugs passively loaded into the intraliposomal aqueous phase, the best loading available to date is $\leq 60\%$. Non-efficient loading leaves a large amount of the drug un-encapsulated, and when the drugs are toxic and/or expensive this un-encapsulated drug is a major drawback. Therefore, an additional step of removal of the free drug is required, which adds unwanted handling and cost to the process of preparation of liposome formulation. A second drawback for methods of solvent or detergent removal is residual level of unwanted solvents or detergents.

The present invention provides a novel, fast and simple method for preparing liposomes efficiently loaded (i.e. at least 60% loading) with ISS-ODN.

Because of the fast clearance/degradation of "free" ISS-ODN after administration, effective encapsulation of the molecule, as achieved by the method of the present

invention, may reduce the number of doses and frequency of administrations required in order to achieve a desired therapeutic effect.

Liposomal ISS-ODN as obtained by the present invention may be utilized, for example, as a vaccine adjuvant against pathogens and cancer; in therapeutic treatment or prevention of diseases caused by certain infectious microorganisms; in the treatment or prevention of allergic diseases; or to boost innate immunity. According to one embodiment, the ISS-ODN is an endotoxin-free ISS-ODN with a phosphorothioate (PS) or phosphodiester (PO) backbone.

According to the invention, liposomal ISS-ODN are prepared by:

(a) solubilizing at least one liposome-forming lipid in a solvent and drying same to effect a dry liposome-forming lipid or a dry mixture of liposome-forming lipids;

(b) providing an aqueous solution of ISS-ODN or of a mixture of ISS-ODNs; and

(c) hydrating the dry liposome-forming lipid or mixture of lipids with the solution of ISS-ODN to yield liposomal ISS-ODN.

As will be shown in the following specific Examples, the method of the invention provides a highly effective entrapment of the biologically active material in the liposomes, typically equal or greater than 60% (from the initial amount of ISS-ODN employed by the method).

According to the present invention the lipids are preferably freeze dried, i.e. by lyophilization thereof, resulting in a powder with a unique arrangement of the lipids enabling the effective loading of the ISS-ODN upon hydration into liposomes formed by the hydration.

The lipids may be any substance that forms liposomes upon dispersion thereof in an aqueous medium. Preferred liposome-forming amphipathic substances are of natural sources, semi-synthetic or fully synthetic molecules; negatively or positively charged phospholipids, sphingolipids, or other lipids optionally combined with a sterol, such as cholesterol, and/or with lipopolymers, such as PEGylated lipids.

The liposome-forming amphipathic substances may include saturated or unsaturated amphiphiles. Non-limiting examples of such amphiphiles are phospholipids including, without being limited thereto, fully hydrogenated, partially hydrogenated or non-hydrogenated soybean derived phospholipids, egg yolk phospholipids, dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG), phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, sphingomeylins, and mixtures of the above.

A preferred phospholipid combination according to the invention includes a mixture of DMPC and DMPG at a molar ratio of DMPC:DMPG between about 1:20 and 20:1. Such mixtures may be combined with cholesterol, and/or PEGylated lipids. PEGylated lipids are commercially available. Preferred PEGylated lipids include, without being limited thereto, DSPE-PEG^{2000} [Haran, G., *et al. Biochim. Biophys. Acta* **1151**:201–215 (1993)] or dihexadecyl phosphatidyl PEG^{2000} (DHP-PEG^{2000}) [Tirosh, O., *et al. Biophys. J.* **74**:1371–1379 (1998); US Patent No. 6,165,501]

The solvent according to the invention is any solvent with which lipids may solubilize or be dissolved. Such solvents include, *inter alia*, the water miscible polar solvent, tertiary-butanol and the water immiscible apolar solvent, cyclohexane.

According to the method of the invention it is advisable to keep the ISS-ODN in a medium isoosmotic to plasma such as 0.9% sodium chloride, or cryprotectant, which is a pharmaceutically acceptable agent such as lactose, sucrose or trehalose or NaCl/cryoprotectant mixture. Thus, the aqueous solution according to the present invention is a physiologically acceptable aqueous medium in which ISS-ODN can solubilize, dissolve, or disperse and may be selected from 0.9% NaCl by weight (Saline), buffered Saline such as phosphate-buffered Saline (PBS), 5% dextrose, buffered dextrose, 10% sucrose and buffered sucrose, or any combination of the same. Alternatively, the ISS-ODN can solubilize in pyrogen-free sterile water (at times referred to as 'water for injection') and after hydration of the dry amphipathic substance, the resulting dispersion is adapted to the physiological conditions suitable for administration.

According to a second aspect, there is provided by the present invention a combination of two compositions, including a first composition comprising dry liposome-forming lipids and a second composition comprising ISS-ODN, the combination is for use in the preparation of a pharmaceutical formulation comprising liposomal ISS-ODN, the preparation being in accordance with the method of the present invention.

The combination of the invention may be obtained in the form of a package. Accordingly, the present invention also concerns a combination at least one first composition of dry liposome-forming lipids; and at least one second composition of ISS-ODN (either dry or in an aqueous solution); in the form of a package further comprising instructions for use of the first and second compositions for the preparation of a pharmaceutical formulation, said instructions comprising hydrating the dry lipid(s) of said first composition with an aqueous solution of ISS-ODN to obtain liposomes loaded with said ISS-ODN (preferably more than 60%); and the package further comprising instructions prescribing administration of said pharmaceutical formulation to a subject in need thereof.

The dry liposome-forming lipids and the ISS-ODN are each contained in a separate vial. The package, according to the invention, may contain more than one of said first composition of dry lipid(s) in separate vials and more than one of second composition comprising ISS-ODN, the instructions for selection and use of the compositions will depend on the specific liposome/ISS-ODN formulation of interest. These instructions may be addressed to the physician, to the pharmacists or even to a subject of the treated individual.

The package may further comprise an aqueous medium, e.g. a physiologically acceptable aqueous medium, in which the ISS-ODN is dissolved or diluted. Alternatively, the aqueous medium may be obtained separately, as it is typically a commercially available medium. Selection of the medium suitable for use will depend on considerations known to those versed in the art and, therefore, do not need to be further discussed herein.

According to one embodiment, the package comprises two or more compositions of dry lipids and optionally two or more compositions of ISS-ODN, thereby enabling to construct different pharmaceutical combinations according to the desired effect to be achieved and instructions prescribed by the medical practitioner. The package may be for use by the physician, by the pharmacist or, at times, by the subject in need of the liposomal formulation.

The invention also concerns pharmaceutical formulations comprising as active ingredient a therapeutically effective amount of liposomal ISS-ODN and optionally a pharmaceutically acceptable additive, the liposomal ISS-ODN being prepared by the method of the invention.

Alternatively, the package may contain at least: (a) one first composition of a dry liposome-forming lipid and (b) liposomal ISS-ODN prepared by the CO method. Mixture (b) may also be in a form of aqueous dispersion.

In fact, the pharmaceutical formulation of the invention is basically the liposomal formulation obtainable by the method of the invention but adapted for administration to the individual in need of a treatment or prevention of an identified disease.

The liposomal ISS-ODN is administered and dosed in accordance with good medical practice, taking into account the nature of the active ingredient, the clinical condition of the individual patient, the site, route and method of administration, scheduling of administration, patient age, sex, body weight and other factors known to medical practitioners.

The liposomal ISS-ODN of the invention may be administered in various ways. It can be administered in combination with pharmaceutically acceptable diluents, excipients, additives and adjuvants, as known in the art, e.g. for the purposes of adding flavors, colors, lubrication or the like to the liposomal formulation.

The pharmaceutically acceptable diluent/s, excipient/s, additive/s employed according to the invention generally refer to inert, non-toxic substances which preferably do not react with the liposomal formulation of the present invention.

Yet, since the ISS-ODN loaded in the liposomes are known to act as immunoadjuvants, the formulation of the invention may comprise other biologically active agents, such as antigens. The additional agents may be in a free form or also encapsulated in liposomes (in the same or different liposomes of the immunoadjuvant) and may be administered simultaneously, concomitant or within a predefined time interval from administration of the immunoadjuvant. Further, the antigen may be, *inter alia*, derived from a killed or modified (e.g. genetically) organism or virus.

The liposomal ISS-ODN can be administered orally, intranasally, or parenterally including intravenously, intraarterially, intramuscularly, intraperitoneally, subcutaneously, intradermally, and intrathecally, and by infusion techniques. Yet further, the liposomal ISS-ODN of the invention may be made into aerosol formulations for administration by inhalation. Such aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like. They also may be formulated as pharmaceuticals for non-pressured preparations, such as in a nebulizer or an atomizer. The manner of administration will depend on different considerations known to the man of the art, e.g. on the type of agent to be loaded into the liposome.

Finally, a method for the prevention or treatment of a disease is also provided by the present invention, the method including administration to a subject in need of liposomal ISS-ODN of the invention.

According to a preferred embodiment, the dosage for the liposomal ISS-ODN include up to 2,000 mg of loaded liposomal vesicles, measured by lipid, per kg body weight of the treatment subject. It should be noted, however, that the accurate dosage can vary dramatically, the variation depends on e.g. the type and efficacy of the ISS-ODN entrapped by the liposome, the efficiency of encapsulation (albeit being high with the method of the invention), the route of administration, the disease to be treated and the like. The respective parameters may be easily optimized by those skilled in the art and can thus be regarded as being routine experiments.

The invention will now be further explained by the following non-limiting examples. While the foregoing description describes in detail only a few specific embodiments of the invention, it will be understood by those skilled in the art that the invention is not limited thereto and that other variations in form and details may be possible without departing from the scope and spirit of the invention as defined by the claims, which are to be read as included within the disclosure of the specification.

SPECIFIC EXAMPLES

General

ISS-ODN-loaded liposomes

The lipids employed for liposome formation in the following examples were DMPC and DMPG, with or without cholesterol. Two types of liposomes containing CpG were formed, for the purpose of comparison of the method of preparation of the present invention with another hitherto known method. The two encapsulation methods employed are designated herein as *post encapsulation* (the method of the present invention) and *co-encapsulation*.

1. ***Post encapsulation***: A lyophilized mixture of the lipids was hydrated with the ISS-ODN, *a priori* dissolved in pure water, and the liposomal preparation was diluted in 0.9% NaCl (Saline) or phosphate-buffered Saline (PBS).
2. ***Co-encapsulation***: The solubilized lipids in tertiary butanol and ISS-ODN in pure water were co-lyophilized overnight and then hydrated with 0.9% Saline.

Materials and Reagents

Influenza subunit vaccines (HN) – Subunit preparations containing mainly the influenza viral surface proteins hemagglutinin (H) and neuraminidase (N), 80-90% and 5-10% (w/w), respectively, derived from strains A/Sydney/5/97 (H3N2), A/Beijing/262/95 (H1N1), A/New Caledonia/20/99 (H1N1), A/Panama/2007/99

(H3N2), and B/Yamanashi/166/98, were provided by Dr's. R. Glück and R. Zurbriggen, Berna Biotech, Bern, Switzerland.

Hepatitis B vaccines (Hb) – Recombinant, yeast (Hansenula polymorpha) derived hepatitis B's antigen (HBsAg, Rhein Biotech, Dusseldorf, Germany, Diminsky *et al.* 1997).

Tuberculosis vaccine –Combined vaccines composed of *M. tuberculosis* derived recombinant proteins (ESAT-6, L7/L12 and 85 B, or Sod, 85B and CFP21) mixed with Ribi adjuvant (Sigma, USA), or without Ribi adjuvant.

Dimyristoyl phosphatidylcholine **(DMPC)** - Lipoid PC 14:0/14:0 562157 (Lipoid GmbH, Ludwigshafen, Germany).

Dimyristoyl phosphatidylglycerol **(DMPG)** - Lipoid PG 14:0/14:0 602035-1 (Lipoid GmbH, Ludwigshafen, Germany).

ODN - Endotoxin-free (1<ng/mg DNA) phosphorothioate ISS-ODN (ODN 1018), (5'-TGACTGTGAACGTTCGAGATGA-3') and the "mutant" M-ODN, lacking the CpG motif (ODN 1019), (5'-TGACTGTGAAGGTTAGAGATGA-3'), both dissolved in distilled water, were obtained from Trilink Biotechnologies, San Diego, CA, USA.

Two additional phosphorothioate ISS-ODNs were obtained from the Weizmann Institute, Rehovot, Israel, ODN 54076 (thioate): TCCAT**AACGTT**GCA**AAC**–**GTT**CTG, and ODN 51997 (thioate): TCCAT**GACGTT**CCT**GACGTT**CTG.

Methods of preparation

Preparation of soluble influenza HN antigen

The subunit preparations were used each separately or combined then diluted in sterile phosphate-buffered saline (PBS pH 7.4) for injection or intranasal administration (0.15–1 μg protein/dose of each viral strain).

Preparation of Liposomal ISS-ODN (Lip ISS-ODN) and stability determination

In the first series of experiments, ISS-ODN (ODN 1018) and M-ODN (ODN 1019) were encapsulated in large (mean diameter 1400±200 nm) multilamellar vesicles (MLV) composed of DMPC and DMPG (DMPC:DMPG, 9:1 mole ratio), at a lipid:ODN ratio of 50:1-500:1 (w/w), under aseptic conditions as follows: The phospholipids were dissolved in tertiary butanol and freeze dried by lyophlization over night. The lipid powder (lipid cake) was then rehydrated at room temperature with the ODN solution. To ensure efficient encapsulation, soluble ODN was added in a minimal volume (e.g. for 10 mg-30mg lipid, 25-50μl of ODN solution was added). This was then vortexed vigorously for about 1 min. until a paste was obtained. The paste was then gradually diluted by further vortexing with sterile PBS or Saline to obtain the required concentration. This method corresponds to the **post encapsulation** method of the present invention.

To determine encapsulation efficiency, the liposomal preparation was centrifuged at 4°C, for 1hr. at 45,000 rpm. The liposome pellet and the supernatant (containing non-encapsulated ODN and traces of small liposomes) were subjected to a 2-phase lipid extraction procedure [Bligh, E.J. and Dyer, W.J. (1959) *Canadian J. Biochem. Physiol.* **37**:911-917]., and the amounts of free and encapsulated ODN and liposomal phospholipids were assessed by organic phosphorus determination [Barenholz, Y. and Amselem, S. (1993) in *Liposome technology*, 2nd ed., Vol I. (Gregoriadis G, ed.), CRC Press, Boca Raton, FL, pp. 501-525 (1993)].

Using the following ratios (w/w) of lipid:ISS-ODN – 50:1, 100:1, 300:1 and 500:1, the mean encapsulation efficiency (of 3 experiments) was 60, 75, 90 and 95%, respectively. No significant ODN leakage (<10%) from the liposomes was found after storage in aqueous dispersion for three months at 4°C. To avoid overloading the mice with extra lipids, which can cause nonspecific immune stimulation [Kedar, E., *et al. J. Immunother.* **23**:131-145 (2000)], the formulation prepared at a 100:1 (w/w) lipid:ODN ratio (mean encapsulation efficiency, 75%) was chosen for vaccination experiments. The free/liposomal ISS-ODN and M-ODN were used at 5-25μg/mouse/dose.

In a second series of experiments, the POST technique described above was compared to another procedure for encapsulating ISS-ODN in liposomes that were prepared by co-lyophilization of a mixture consisting of the solubilized lipids (in tertiary butanol) and ISS-ODN in aqueous solution, followed by hydration with 0.9% NaCl(referred to herein as the **co-encapsulation** method).

In these experiments (Table 1), ODN 1018 and the influenza antigens (HN) were encapsulated, each separately or combined, in liposomes composed of DMPC alone, DMPC/DMPG (9:1 molar ratio), or DMPC/cholesterol (6:4 molar ratio), using the post- or the co-encapsulation techniques at a lipid/ODN w/w ratio of 100/1, and at a lipid/protein(HN) w/w ratio of 300/1. Both techniques resulted in 60–80% encapsulation of ODN 1018 in all lipid compositions tested, when separate vesicles were used for ISS-ODN and the influenza antigens. For co-encapsulation of ODN 1018 and HN within the same vesicles, the CO technique was superior over the POST technique (70% vs 30% ODN 1018 encapsulation).

Table 1-Encapsulation and co-encapsulation of influenza antigens (HN) and ISS-ODN 1018 in various liposomal formulations (MLV) prepared by the POST or the CO technique

Formulations	**Agent**	**Encapsulation method for ODN**	**% HN encapsulation**	**% ODN encapsulation**
DMPC				
Same vesicles	HN+ODN	POST	52-80	3-28
		CO	62	52-56
Separate vesicles	HN+ODN	POST	80-100	67-89
		CO	100	42-51
	HN alone	POST	87-93	-
	ODN alone	POST	-	62-63
		CO	-	61-66
DMPC/DMPG (9:1)				
Same vesicles	HN+ODN	POST	50-80	14-24
		CO	66	78-82
Separate vesicles	HN+ODN	POST	80-100	72-86
		CO	100	79-80
	HN alone	POST	80-100	-
	ODN alone	POST	-	61-66
		CO	-	66-67
DMPC/Chol (6:4)				
Same vesicles	HN+ODN	POST	NT	14-22
		CO	NT	69-77
Separate vesicles	HN+ODN	POST	NT	52-64
		CO	NT	63-82
	HN alone	POST	NT	-
	ODN alone	POST	-	64
		CO	-	63

The data presented in Table 1 is a compiled summary of 3 separate experiments, using subunit vaccines derived from influenza A/Panama/2007/99 (H3N2) and B/Yamanashi/166/98 strains.

According to the POST-encapsulation method, the lipids dissolved in tertiary butanol and lyophilized overnight. The dried lipid powder was then hydrated by stepwise addition (in 25-50μl aliquotes) and vortexing of HN and/or ODN solution. According to the CO-encapsulation method, the solubilized lipids (solubilized in tertiary butanol as described above) are mixed with the ODN solution and co-lyophilized overnight, then hydrated stepwise (as above) with the HN solution or saline. Due to HN instability upon lyophilization, HN was encapsulated using the POST technique only. The lipid/ODN (w/w) ratio was 100/1, and the lipid/HN (w/w) ratio was 300/1.

Mixing of preformed empty liposomes (any of the formulations) in aqueous (saline) suspension with HN and ODN resulted in encapsulation (association) efficiency of 40-60% and 1-3%, respectively.

In a third series of experiments, an approximately 70% encapsulation was obtained at a lipid: ODN w/w ratio of 100:1 using ISS-ODN tagged with the fluorescent marker FITC. Liposomes were pelleted and extracted as above and the level of CpG encapsulation in liposomes was determined by two methods: organic phosphorus determination (described above) and from the FITC fluorescence intensity at 525 nm (excitation 495 nm).

In a fourth series of experiments, liposomal ISS-ODN was prepared at 100:1 and 300:1 lipid: ODN w/w ratios using saline or citrate buffer (containing 150 mM NaCl in 5 mM sodium citrate) pH 6.5–6.7 as final media for storage. The preparations were stored for up to 5 months at 4°C and at room temperature. ISS-ODN leakage and liposome lipid stability were tested 1, 3 and 5 months after preparation. The lipids in the dried lipid "cake" used to prepare the liposomal vaccine by the "post" encapsulation method were found to be chemically stable when stored at 4°C for at least 2 years.

At a lipid: ODN w/w ratio of 100:1 there was a ≤5% ODN leakage at 1 month and 5-15% leakage at 3 and 5 months when stored in unbuffered saline at 4°C and in citrate buffer at both 4°C and room temperature. Approximately 30% release was noted in the saline preparation at room temperature after 3 months. Thus, the liposomal formulation as aqueous suspension is very stable (at least for 5 months at 4°C).

Liposome lipid stability was determined by thin layer chromatography (TLC) and by the release of non-esterified fatty acids (NEFA) as a result of aryl ester hydrolysis. No significant lipid degradation (<5% hydrolysis) was noted up to 3 months at both temperatures for both saline and citrate buffer. A marked degradation (>10%) was found at 5 months, but only in unbuffered saline at room temperature. Much lower level of degradation was found in saline/citrate buffer than in unbuffered saline. Formulations prepared at a lipid:ODN w/w ratio of 300:1 were slightly less stable and leakier than those prepared at a 100:1 ratio.

Preparation of Liposomal HN (Lip HN)

HN-loaded large multilamellar vesicle (MLV) liposomes (mean diameter, 1.5 μm) were prepared by using the **POST-encapsulation** method as described above in connection with preparation of Lip ISS-ODN by hydrating the dry lipid cake with the HN solution. In addition, HN was encapsulated with the ISS-ODN in the same vesicles using the POST technique for HN and the POST or CO technique for ISS-ODN, as summarized above (Table 1).

In short, vials of 100 mg of DMPC:DMPG (9:1 molar ratio), DMPC alone, or DMPC/cholesterol (6:4 molar ratio) solubilized in tertiary-butanol, were frozen and then lyophylized over night to form the dry lipid cake. Upon need, the dry lipid was hydrated with a mixture of the subunit (HN) preparations (derived from 1, 2, or 3 viral strains, see materials and reagents) by adding the soluble HN subunits at a lipid:HN ratio of 300:1 (w/w) in increments of 50 μl and vortexing vigorously. Liposomes co-loaded with HN and ISS-ODN 1018 were prepared by (a) hydration of the dry lipids, performed as above, with an aqueous solution mixture containing HN + ISS-ODN 1018 (the POST

technique), (b) co-lyophilization of the lipid + ISS-ODN solution (CO), followed by hydration with the HN solution (POST). The liposomes were then further diluted in sterile saline or PBS and stored at 4°C.

Encapsulation efficiency was assessed by spinning the loaded liposomes at 4°C for 30 min. at 14,000 rpm (under these conditions free HN does not precipitate while the majority of the MLV liposomes do), and by determining the protein concentration of the supernatant and of the liposomal fraction using a modified Lowry determination assay [Peterson G.L., Methods Enzymol. **91**:95-119 (1983)]. HN Encapsulation in separate vesicles or in vesicles also loaded with ISS-ODN was found to be 60-90%, depending on viral stain, using the two procedures described above. ODN encapsulation was determined as described above and it was 60–80% when encapsulated alone by both procedures or when encapsulated together with HN in the same liposomes using procedure (b) described above. ISS-ODN encapsulation was 20–30% when encapsulated together with HN using procedure (a) described above (Table 1).

For both Lip ISS-ODN and Lip HN, the lipid integrity was found to be very high (>95%) by analyzing samples of the lipids by thin layer chromatography (TLC).

Example 1 - *Enhancement of the systemic anti-viral humoral response by liposomal ISS-ODN (Lip ISS-ODN) co-administered intramuscularly (i.m.) with influenza subunit vaccines*

Female Balb/c mice, 6-8-weeks-old (5-6 per group) were vaccinated once (0.1 ml) i.m, with a divalent influenza subunit vaccine composed of the viral surface proteins hemagglutinin and neuraminidase (HN) derived from the A/Beijing/262/95-like (H1N1) and B/Yamanashi/166/98-like strains (0.15 μg protein of each strain). The HN antigens were given either in soluble form or entrapped in liposomes (Lip HN), alone or in combination with free ISS-ODN (ODN 1018) or Lip ISS-ODN (5, 12.5, 25 μg, Table 2). The liposomes comprised DMPC:DMPG (9:1 mole ratio), and HN and ISS-ODN were encapsulated by the POST technique. Preparation of the Lip HN and Lip ISS-ODN is described herein before.

Two additional groups (group 9 and group 10, Table 2) were immunized with liposomes co-entrapping the HN antigens and the ISS-ODN. Mutant ODN (M-ODN) lacking the immunostimulatory sequence served as control. Sera were tested 3 weeks (21 days) and 3 months (90 days) postvaccination for hemagglutination-inhibiting (HI) Abs [Sever, J. (1962). *J Immunol.* **88**:321-325] (Table 2) and for antigen-specific IgG1 and IgG2a Abs (Table 3). In the latter test, antigen-specific isotypes were tested 21 and 90 days postvaccination on pooled sera from each group (starting at a 1/10 dilution) by ELISA [Babai *et al.* Vaccine **17**:1239-1250 (1999)]. The antibody (Table 3) titer is expressed as the reciprocal of the highest serum dilution yielding 50% of the maximum absorbance obtained with "standard" immune serum, after subtracting the control (antigen + normal mouse serum).

Table 2-The anti-hemagglutinin response (HI titer) following vaccination with free/liposomal divalent influenza vaccine

Vaccine and dose	HI titer (mean±SD) against: A/Beijing (Day 21) [a]	B/Yamanashi (Day 21) [a]	A/Beijing (Day 90) [a]	B/Yamanashi (Day 90)
1. HN 0.15 μg	20±14 (20%)	6±5 (0%)	128±44 (100%)	12±18 (20%)
2. Lip HN	56±22 (100%)	24±17 (40%)	208±107 (100%)	48±30 (60%)
3. HN+ISS-ODN 5μg	28±11 (40%)	20±14 (20%)	160±150 (80%)	26±31 (20%)
4. HN+ISS-ODN 12.5 μg	24±17 (40%)	20±12 (20%)	44±36 (60%)	12±18 (20%)
5. HN+ISS-ODN 25 μg	20±20 (40%)	16±9 (0%)	82±76 (60%)	18±13 (20%)
6. HN+Lip ISS-ODN 5 μg [b]	128±44 (100%)	80±0 (100%)	352±175 (100%)	68±11 (100%)
7. HN+Lip ISS-ODN 12.5 μg [b]	96±36 (100%)	72±18 (100%)	288±72 (100%)	96±61 (100%)
8. HN+Lip ISS-ODN 25 μg [b, c]	176±88 (100%)	144±36 (100%)	480±225 (100%)	144±36 (100%)
9. Lip (HN+ISS-ODN 5 μg)	160±0 (100%)	72±18 (100%)	448±175 (100%)	72±18 (100%)
10. Lip (HN+ISS-ODN 25 μg)	144±36 (100%)	128±44 (100%)	448±175 (100%)	104±54 (100%)
11. HN+M-ODN 25 μg	14±19 (20%)	8±8 (0%)	106±136 (60%)	8±11 (0%)
12. HN+Lip M-ODN 25 μg	30±10 (40%)	22±11 (20%)	176±88 (100%)	20±14 (20%)

[a] In parentheses, % seroconversion = % of mice achieving a HI titer ≥40.

[b] Groups 6, 7, 8 vs. groups 3, 4, 5, $P < 0.05$ (t-test) for HI titer at both time points against the two viruses.

[c] Group 8 vs. group 12, $P \leq 0.01$ for HI titer at both time points against the two viruses.

Table 3 - Anti-HN IgG isotypes following vaccination with a divalent influenza vaccine co-administered with free/liposomal ISS-ODN

Vaccine	Mean IgG titer against							
	A/Beijing d.21		B/Yamanashi d.21		A/Beijing d.90		B/Yamanashi d.90	
	IgG1	IgG2a	IgG1	IgG2a	IgG1	IgG2a	IgG1	IgG2a
1. HN 0.15 μg	<10	<10	20	<10	70	20	100	80
2. Lip HN	20	<10	80	<10	110	80	190	100
3. HN+ISS-ODN 5 μg	<10	70	<10	100	<10	350	30	450
4. HN+ISS-ODN 12.5 μg	<10	32	<10	80	<10	500	<10	380
5. HN+ISS-ODN 25 μg	<10	64	<10	70	<10	350	<10	450
6. HN+Lip ISS-ODN 5 μg	<10	450	<10	600	20	2500	<10	5000
7. HN+Lip ISS-ODN 12.5 μg	<10	560	<10	400	<10	2500	<10	4200
8. HN+Lip ISS-ODN 25 μg	<10	400	<10	600	<10	2400	<10	4600
9. Lip (HN+ISS-ODN 5 μg)	<10	400	<10	500	<10	2000	<10	4200
10. Lip (HN+ISS-ODN 25 μg)	<10	550	<10	800	10	1800	<10	5000
11. HN+M-ODN 25 μg	<10	<10	<10	<10	190	50	200	20
12. HN+Lip M-ODN 25 μg	<10	<10	<10	<10	300	20	300	10

Table 2 shows the results of hemagglutination inhibition (HI), while Table 3 shows the results of the ELISA of antigen-specific IgG1 and IgG2a Abs.

As can be seen in Table 2, the HI titer, as well as the percent seroconversion, of mice co-immunized with Lip ISS-ODN (groups 6-8) were significantly greater than those of mice vaccinated with antigen alone (groups 1, 2) or with antigen combined with soluble ISS-ODN (groups 3-5). The HI titers obtained with Lip ISS-ODN were 2-8-fold higher than with soluble ISS-ODN. At 3 weeks postvaccination, the seroconversion (HI titer $\geq$40) rate was 0-40% using soluble ISS-ODN or M-ODN and 100% with Lip ISS-ODN. The superior adjuvant activity of Lip ISS-ODN was seen at all doses, at both time points, and for the two viral strains. The response attained at the lowest ISS-ODN dose (5 μg) was similar to that obtained with the highest dose (25 μg). Vaccination with liposomes loaded with the antigen and the ISS-ODN in the same vesicles (groups 9, 10) (using the post technique for both) produced a response similar to that achieved with a mixture of Lip ISS-ODN and the free antigen (groups 6-8). However, it is worth noting that under such conditions (i.e., mixing of preformed liposomes with free HN) approximately 40% of the antigen became liposome associated. Similar results were also obtained with a mixture of Lip ISS-ODN and Lip HN (data not shown). M-ODN, free and liposomal, had no adjuvant effect (groups 11, 12), indicating the need for the CpG sequence for the adjuvant activity.

Assessment of antigen-specific IgG1 and IgG2a (Table 3) showed a modest increment of IgG2a (and abrogation of IgG1) by soluble ISS-ODN (groups 3-5) and a dramatic increase in IgG2a by Lip ISS-ODN either mixed with free antigen (groups 6-8) or co-entrapped with the antigen (groups 9, 10). The IgG2a levels produced by Lip ISS-ODN were 4-18 times greater than with soluble ISS-ODN. M-ODN, free and liposomal, slightly increased the IgG1 level and reduced the IgG2a level at 3 months post-vaccination (groups 11, 12).

Thus, a single vaccine dose containing even 5 μg of Lip ISS-ODN results in very high HI titers and specific IgG2a levels, with no IgG1 response, namely a Th1 type. This enhanced response lasts for at least 3 months. The results of this experiment were reproduced in two additional experiments, using divalent and trivalent (adding the

A/Sydney/5-97-like, H3N2, to the divalent preparation) vaccines. The HI titers on days 21-90 were 4-10-fold higher with Lip ISS-ODN (10 μg) than with non-liposomal ISS-ODN. Moreover, Lip ISS-ODN 1018 prepared by the POST technique and Lip ISS-ODN prepared by the CO technique had an equal adjuvant activity following intramuscular co-administration with the influenza HN antigens (data not shown).

In addition to testing the primary response, some groups (groups 5, 6) received a second vaccine dose 3 months after the primary dose (i.e. vaccination on days 0 and 90) and sera were tested 7 days later. The results of this additional test are presented in the following Table 4.

Again, as seen in the primary response, repeated co-vaccination with Lip ISS-ODN produced HI titers 3.3-5 greater and IgG2a levels 3.3-8 higher than with soluble ISS-ODN. No increase in IgG1 was seen with either formulation. These results indicate that Lip ISS-ODN preserves its adjuvant activity also with repeated administration.

Table 4- Secondary immune response of mice immunized with free/liposomal divalent influenza vaccine

Vaccine	HI titer (mean ± SD) against:		Mean IgG titer against:			
	A/Beijing	B/Yamanashi	A/Beijing		B/Yamanashi	
			IgG1	IgG2a	IgG1	IgG2a
HN 0.15 μg	320±0 (100%)	260±150 (100%)	500	<10	1000	200
HN+ISS-ODN 25 μg	896±350 (100%)	368±260 (100%)	400	1,000	600	3,000
HN+Lip ISS-ODN 25 μg	3000±1500 (100%)	1900±800 (100%)	600	8,000	1,000	10,000
HN+M-ODN 25 μg	640±0 (100%)	170±200 (50%)	500	<10	800	100

5

Induction of DTH response by Lip ISS-ODN 1018

In addition to assessing the systemic humoral response, some groups that were vaccinated twice at a 3-month interval were tested for DTH response following subcutaneous (s.c.) injection of hemagglutinin. In particular, 7 days after the second vaccine dose 10 μg of A/Beijing hemagglutinin was injected s.c. into the hind footpad, and footpad swelling was measured at 24 hrs. The increase in footpad thickness (mm) was determined by comparison to the footpad thickness prior to hemagglutinin injection. As control, the contralateral foot was injected with PBS (increase in footpad thickness at 24 hrs. for PBS was 0.037±0.05 mm). The results are shown in the following Table 5.

Table 5-DTH response following vaccination with a divalent influenza vaccine co-administered with free/liposomal ISS-ODN

Vaccine	**Increase in footpad thickness (mm)[a] Mean ±SD**	**Incidence of mice with DTH[b]**
1. Lip HN	0.06±0.01	0/3
2. HN+ISS-ODN 5μg	0.12±0.08	2/5
3. HN+Lip ISS-ODN 5 μg	0.16±0.09	2/5
4. Lip (HN+ISS-ODN 5 μg)	0.52±0.2	5/5
5. HN+M-ODN 25μg	0.04±0.03	0/3

[a] P values: group 4 vs. all groups ≤ 0.004.; group 2 vs. group 3 >0.05 (t-test).

[b] An increase in footpad thickness greater than 10%, relative to time 0, was considered a positive DTH response.

As can be seen from Table 5, a significant DTH response in all mice (5/5) was observed in group 4 vaccinated with liposomes encapsulating both the antigen and the ISS-ODN (5 μg). Mice immunized with soluble antigen mixed with 5 μg of soluble or liposomal ISS-ODN (groups 2,3) developed a moderate response, and only 2/5 were considered positive. Vaccination with antigen alone or with antigen coadministered with M-ODN (25 μg) did not induce a response.

DISCUSSION

The above results demonstrate that ISS-ODN 1018 can be encapsulated in large multilamellar liposomes with high efficiency (up to 95%) using the "post" technique, and that the liposomal formulation is a considerably more potent parenteral adjuvant in mice than the soluble form of ISS-ODN.

A single dose of 5 μg Lip ISS-ODN was by far more potent (4-11 times, Tables 2,3) for the two viral strains than 25 μg of free ISS-ODN. The enhanced potency was reflected in both humoral and cellular (DTH) responses. As with free ISS-ODN, Lip ISS-ODN mainly enhanced Th1-biased immunity, based on the relative IgG1 and IgG2a levels upon i.m. administration.

Further, the results disclosed herein and additional results (data not shown) show that the same increment in the humoral response was obtained when free or liposomal antigen (HN) was administered together i.m. with Lip ISS-ODN (in separate liposomes), and when the vaccine consisted of antigen and ISS-ODN entrapped together in the same liposomes using the post technique. These results differ from those reported recently by two groups on liposomal plasmid DNA and liposomal ISS-ODN. Gursel et al. [Gürsel, M., et al. (1999) Vaccine. 17:1376-1383] found that the adjuvant activity of a non-coding plasmid was demonstrated only when the plasmid and the hepatitis B surface antigen were entrapped together in the same liposomes, but not in separate liposomes. Ludewig et al. [Ludewig, B., et al. (2001) Vaccine. 19:23-32] showed that the anti-viral and anti-tumor responses following vaccination with liposome-entrapped peptides could be augmented by ISS-ODN. However, the response obtained with liposomal antigens admixed with non-liposomal ISS-ODN was similar to that produced by antigens and ISS-ODN incorporated into the same liposomes. This is not the case in our study; our data (Tables 2,3) show that ISS-ODN was an equally effective adjuvant (for the humoral response) when entrapped in the same liposomes with the antigen or in separate liposomes, and Lip ISS-ODN was always more effective than free ISS-ODN (5-25 μg). These differences appears to result from (a) the different and more efficient procedure of the present invention for the encapsulation and delivery of ISS-ODN, and the different chemical composition and size of the liposomes, both of which may affect the

localization of the ISS-ODN in the liposome and its delivery; and maybe also from (b) differences in the experimental systems, including the antigens and the type of ISS-ODNs, the route of administration, the dosage, and the assays used to monitor the response.

It should be noted, however, that in contrast to the effect of Lip ISS-ODN on the systemic humoral response, the DTH response (Table 5) was greater when the antigen and the ISS-ODN were encapsulated together in the same liposomes, as compared with the DTH response obtained with liposomal antigen mixed with liposomal ISS-ODN (in separate liposomes).

The superior adjuvant activity of Lip ISS-ODN over free ISS-ODN for intramuscular influenza vaccine was not limited to ODN 1018. In another experiment ISS-ODN 1018 was compared with two other ISS-ODNs containing CpG motifs. All three ISS-ODNs exhibited a similar adjuvant activity, and Lip ISS-ODN was 2–7 times more potent (for HI titer and IgG2a titer) than the corresponding free ISS-ODN (Table 6). According to this specific experiment, BALB/c mice (n=4, group 7, 3 mice/group) were injected once i.m. with 0.5 μg of a subunit vaccine derived from influenza A/New Caledonia/20/99 (H1N1), alone and combined with 10 μg of free or liposomal ISS-ODN. Hemagglutination inhibition (HI) was tested on individual sera and Ig were tested by ELISA on pooled sera. The liposomes (MLV) were composed of DMPC/DMPG (9:1 mole ratio) and ODN was encapsulated by the POST technique at a 100/1 lipid/ODN w/w ratio.

Table 6-Comparison of different ISS-ODNs as adjuvants for influenza vaccine: HI, IgG1 and IgG2a titers 4 weeks post vaccination

Vaccine	Mean HI titer[a]	Mean IgG1 titer	Mean IgG2a titer	IgG2a/IgG1 ratio
1. None	5 (0)	<10	<10	-
2. HN alone	9 (0)	1500	60	0.04
3. HN + free ODN 1[b]	52 (75)	900	1500	1.7
4. HN + lip ODN 1	140 (100)	2000	2800	1.4
5. HN + free ODN 2[c]	31 (50)	45	700	15.5
6. HN + lip ODN 2	210 (100)	1500	3500	2.3
7. HN + free ODN 3[d]	57 (33)	65	4500	69.2
8. HN + lip ODN 3	180 (100)	2800	11000	3.9

[a] In parenthesis, % seroconversion (% of mice with an HI titer ≥ 40).

[b] ODN 54076 (thioate);

[c] ODN 51997 (thioate);

[d] ODN 1018 (thioate).

Enhancement of mucosal anti-viral response and protective immunity by Lip ISS-ODN co-administered intranasally (i.n.) with influenza vaccine

A liposomal divalent vaccine (Lip HN, 0.5 μg of each strain) was administered twice i.n., spaced 1 week, alone and in combination with free or Lip ISS-ODN (10 μg), or with free cholera toxin (CT, 1 μg), considered a powerful (yet toxic) mucosal adjuvant. In this case, HN and ISS-ODN were incorporated into the same DMPC/DMPG liposomes by the POST technique. Serum, nasal wash and lung homogenate were tested for antigen-specific IgG1, IgG2a and IgA Abs four weeks after the second vaccine dose, and protective immunity to viral challenge was assessed six weeks post-vaccination by determining lung virus titer. The mean Ig titer was tested by ELISA on pooled samples. Values obtained with normal mouse serum were subtracted. Lungs were washed x3 in cold PBS then homogenized in 1.5 ml PBS per lungs of each mouse (referred to as 1/10 dilution). The homogenates were then centrifuged at 3000 rpm for 30 min at 4°C, the supernatants were collected and centrifuged at 14,000 rpm for 20 min at 4°C, and

antibody titer in the supernatants was measured. Nasal wash was made in 0.2 ml PBS (referred to as 1/10 dilution). Serial 2-fold dilutions were tested, starting with 1/20 sample dilution. The results are shown in the following Table 7. The ELISA antibody titer was determined as described for Table 3.

Table 7 -Serum and mucosal anti-HN IgG1, IgG2a and IgA antibodies 4 weeks following intranasal vaccination with a liposomal divalent influenza vaccine co-administered with free/liposomal ISS-ODN

Vaccine (n = 5/group)	Location	Mean titer against					
		A/Beijing			B/Yamanashi		
		IgG1	IgG2a	IgA	IgG1	IgG2a	IgA
Lip HN	Serum	<20	<20	<20	<20	<20	<20
Lip HN+free ISS-ODN		<20	<20	<20	<20	40	<20
Lip (HN+ISS-ODN)		45	65	<20	100	300	90
Lip HN+CT		30	75	<20	300	100	50
Lip HN	Nasal wash	<20	<20	<20	<20	<20	<20
Lip HN+free ISS-ODN		<20	<20	<20	<20	<20	<20
Lip (HN+ISS-ODN)		90	55	110	90	60	60
Lip HN+CT		275	20	20	150	50	50
Lip HN	Lung homogenate	<20	<20	<20	<20	<20	25
Lip HN+free ISS-ODN		<20	<20	<20	<20	<20	<20
Lip (HN+ISS-ODN)		<20	70	200	<20	20	60
Lip HN+CT		475	55	200	350	75	20

As shown in Table 7, soluble ISS-ODN had no adjuvant effect (except for a low increase in serum IgG2a against one viral strain). In contrast, Lip ISS-ODN induced significant levels of antigen-specific IgG1, IgG2a and IgA in serum, nasal wash and lung. The response to the two viral strains included in the vaccine varied, however. Lip ISS-ODN and CT elicited similar levels of serum and mucosal IgG2a and IgA; the latter inducing higher levels of IgG1 as well. Thus, under the experimental conditions used, Lip ISS-ODN, but not soluble ISS-ODN, is capable of boosting both systemic and mucosal antigen-specific IgG2a and IgA Abs.

The remaining mice of this experiment (5 per group) were challenged i.n. with a recombinant A/Beijing virus. In particular, six weeks after the second vaccine dose, mice were lightly anesthetized with Halotane and 25 μl live virus suspension per nostril was administered. The test virus was the reassortant virus X-127 (A-Beijing/262/95 (H1N1) x X-31 (A/Hong Kong/1/68 x A/PR/8/34), which is infectious to mice, 10^7 EID 50 (egg-infectious dose 50%). The mice were sacrificed on day 4 post-infection, the lungs were removed, washed x 3 in cold PBS, and homogenates in PBS of each group were pooled and centrifuged at 2000 rpm for 30 min. at 4°C and the supernatants collected. Serial ten-fold dilutions were performed and 0.2 ml of each dilution was injected in duplicate into the allantoic sac of 10-11 days-old fertilized chicken eggs. After 48 hrs., at 37°C the eggs were stored overnight at 4°C. Then 0.1 ml of allantoic fluid was removed and checked for viral presence by hemagglutination with chicken erythrocytes (0.1 ml of 0.5% suspension) for 30 min at room temperature. The lung virus titer is determined as the highest dilution of lung homogenate producing virus in the allantoic fluid (positive hemagglutination). A titer of 0.5 indicates 1 egg was positive and 1 egg was negative at the highest dilution.

Table 8-Protection against influenza virus infection 6 weeks following intranasal vaccination with a divalent influenza vaccine co-administered with free/liposomal ISS-ODN

Vaccine (n=5)	**Lung virus titer (log10)**
Unimmunized	6
Lip HN	5
Lip HN + free ISS-ODN	4
Lip (HN+ISS-ODN)	2.5
Lip HN + CT	3

Table 8 shows that, as compared with unimmunized mice, the lung virus titer of mice vaccinated with Lip HN alone, Lip HN+free ISS-ODN and Lip (HN+ISS-ODN) was reduced by 1, 2 and 3.5 logs, respectively (and 3 logs with CT). The 30-fold difference in lung virus titer between free and liposomal ISS-ODN is in good agreement with the differences seen in HI, IgG2a and IgA titers between these groups.

In another intranasal experiment (Table 9), a comparison was made between Lip ISS-ODN 1018 encapsulated by the POST technique and Lip ISS-ODN 1018 encapsulated by the CO technique. According to this experiment, BALB/c mice (n=5) were vaccinated i.n. on days 0, 7 with a subunit vaccine derived from influenza A/New Caledonia/20/99 (H1N1). In groups 6,7 separate vesicles were formed for HN and ODN 1018; in groups 8, 9 HN and ODN 1018 were encapsulated together within the same vesicles. In all formulations, HN was encapsulated by the POST technique. Liposomes (MLV) consisted of DMPC/DMPG (9:1 mole ratio). Response was measured 4 weeks after the second vaccine dose. CT-cholera toxin.

In most cases (Table 9, groups 6–9) both liposomal ISS-ODN formulations showed a similar adjuvant activity, except for the superior activity of Lip ISS-ODN formed by the CO technique upon administration together with free HN (groups 4,5). Moreover, Lip ISS-ODN was superior to free ISS-ODN with regard to HI and IgG2a titers.

Table 9-The anti-influenza humoral response of BALB/c mice vaccinated intranasally with various liposomal formulations of HN and ISS-ODN 1018

Vaccine	Encapsulation method of ODN	Mean HI titer[a]	Mean IgG1 titer[b]	Mean IgG2a titer[c]
1. None	-	<10 (0)	<10	<10
2. HN 1 μg	-	<10 (0)	15	<10
3. HN+free ODN 10 μg	-	<10 (0)	400	100
4. HN+lip ODN	POST	<10 (0)	190	210
5. HN+lip ODN	CO	27 (60)	550	350
6. Lip HN +lip ODN	POST	26 (60)	1100	160
7. Lip HN +lip ODN	CO	12 (20)	1000	300
8. Lip (HN+ODN)	POST	24 (40)	350	300
9. Lip (HN+ODN)	CO	22 (40)	650	300
10. HN+CT 1μg	-	120 (100)	25000	400

[a]Tested by hemagglutination inhibition (HI) on individual sera. In parenthesis, % seroconversion (% of mice with an HI titer ≥40).

[b]Tested by ELISA on pooled sera. The titer was determined as the highest serum dilution yielding OD=0.2.

In an additional experiment both the humoral and cellular responses were tested in parallel following parenteral vaccination with a subunit influenza vaccine co-administered with free or liposomal (lip) ISS-ODN.

BALB/c mice were immunized once intramuscularily (i.m.) with a monovalent subunit vaccine (0.5 μg) derived from A/New Caledonia/20/99-like (H1N1), alone and combined with 10 μg of free or lip ISS-ODN (MLV liposomes (1.5 μm) consisted of DMPC:DMPG at 9:1 mole ratio, lipid:ODN w/w ratio of 100:1 and prepared as described above). Mice injected with lip ISS-ODN alone served as control. Humoral (hemagglutination inhibition [HI] titer, serum and lung IgG1, IgG2a, IgA and IgE titer, Table 10) and cellular (proliferation, cytokine production, cytotoxicity, Table 11) responses were assessed 4 and 6 weeks post-vaccination using spleen cells.

For determining cellular response, cells (0.5 x 10^6/well) were incubated in U-shaped 96-well plates, in triplicate, with or without the HN antigen (0.5 μg/well), in a final volume of 0.2 ml of RPMI 1640 medium supplemented with 5% FCS. After 72 h, cultures were pulsed with 1 μC ^{3}H-thymidine for 16 h.

For determination of IFNγ production cells, (2.5 x 10^6/well) were incubated in 24-well plates, in duplicate, with or without the HN antigen (10 μg/well), in a final volume of 1 ml of RPMI 1640 + FCS. Supernatants were collected after 72 h and tested by ELISA for murine IFNγ and IL-4.

For determining cytotoxic activity, responding splenocytes, (2.5 x 10^6/well) were incubated in 24-well plates, in duplicate, with or without the HN antigen (10 μg/well) in a final volume of 1 ml of RPMI 1640 + 10% FCS + 5 x 10^5M 2-mercaptoethanol. On day 7 cytotoxicity was tested in a 4 h ^{51}Cr release assay (at an effector/target cell ratio of 15/1, in triplicate) against labeled non-infected P815 cells and P815 cells infected with the reassortant virus X-127 (H1N1, which is infectious to mouse cells and cross-reactive with A/New Caledonia), using 0.2 ml of virus-containing allantoic fluid for 5x10^6 cells, for 2 h at 37°C. Specific cytotoxicity was calculated after subtracting the cytotoxicity against non-infected P815.

Table 10-The humoral response of BALB/c mice immunized i.m. with a monovalent influenza subunit vaccine, alone and combined with free/liposomal ISS-ODN

HN vaccine [a] (n=4)	Serum HI titer (Mean ± SD) at 4 weeks [b]	Serum IgG1 titer (Mean) at 6 weeks [c]	IgG2a IgG2 titer (Mean)	Lung homogenate IgG1 titer (Mean) at 6 weeks [c]	Lung homogenate IgG2a titer (Mean)
1. None	<10 (0)	<10	<10	<10	<10
2. HN (0.5 μg) alone	<10 (0)	600	<10	75	<10
3. HN + ISS-ODN (10 μg)	80±0 (100)	150	150	<10	25
4. HN + lip ISS-ODN (10 μg)	320±113 (100)	300	1500	<10	150
5. Lip ISS-ODN alone	<10 (0)	<10	<10	<10	<10

[a] prepared as described;

[b] Mice were tested individually, in parentheses, % seroconversion (% of mice with HI titer ≥40);

[c] Tested by ELISA on pooled samples starting at 1/10 dilution. The reciprocal of the highest sample dilution yielding 50% of the maximum absorbance obtained with a "standard" immune serum was considered the IgG1 or IgG2a titer.

Table 11-The cellular response of BALB/c mice immunized i.m. with a monovalent influenza subunit vaccine, alone and combined with free/liposomal ISS-ODN

Vaccine[a] (n=4)	Proliferative response (stimulation index)[b]	IFNγ production (pg/ml)	% Specific cytotoxicity (E/T ratio = 15/1) [cm]
1. None	1.7	<10	2
2. HN (0.5 μg) alone	3.1	60	9
3. HN + ISS-ODN (10 μg)	2.2	50	7
4. HN + Lip ISS-ODN (10 μg)	9.3	220	37
5. HN + Lip M-ODN (10 μg)	4.3	20	6
6. Lip ISS-ODN alone	1.0	<10	6

[a] Vaccine prepared as described above;

[b] Stimulation index = mean cpm of cells cultured with antigen/mean cpm of cells cultured without antigen;

[c] The values presented were obtained in antigen-containing cultures; no significant cytotoxicity (<5%) was seen in cells cultured without antigen. Cytotoxicity against non-infected p815 target cells was subtracted.

As can be seen in Table 10, no HI response was induced at 4 weeks by antigen alone (HN, hemagglutinin + neuraminidase) or by lip ISS-ODN alone. Mice co-immunized with HN and free/lip ISS-ODN showed a 100% seroconversion (HI titer ≥ 40); however, the HI titer obtained with lip ISS-ODN was 4 times greater than that with free ISS-ODN. Testing by ELISA of antigen-specific immunoglobulin isotypes in serum and lung homogenates at 6 weeks post-vaccination, again showed no response to antigen alone and a moderate increase of IgG2a by co-administered free ISS-ODN. Using lip ISS-ODN as an adjuvant, the serum and lung IgG2a levels were 10 and 6 times higher, respectively, than those achieved with free ISS-ODN.

The presence of a relatively high level of IgG2a antibodies in the lung following parenteral vaccination with lip ISS-ODN is of particular interest. Lip ISS-ODN alone had no effect. No increase in IgG1 was seen with either formulation of ISS-ODN. Antigen-specific IgA and IgE were undetectable. Thus, for enhancing the humoral response, lip ISS-ODN (10 μg) is 4-10 times more efficient than free ISS-ODN.

With regard to cellular responses (Table 11), free ISS-ODN had no adjuvant activity in any of the three tests performed. IFNγ (≥20 pg/ml) was detected only in cultures containing the antigen. No IL-4 (<20 pg/ml) was detected in any of the groups. As compared with free ISS-ODN, *in vitro* stimulated splenocytes of mice co-immunized with antigen and lip ISS-ODN exhibited a 4.2-, 4.4- and 5.3-fold greater proliferative response, interferon γ production and specific cytotoxic activity, respectively. As control, stimulation of naive splenocytes with concanavalin A (2.5 μg/well) produced 2000 pg/ml of IFNγ and 70 pg/ml of IL-4 (data not shown). This experiment indicates that, in addition to its superior adjuvant activity for humoral responses, lip ISS-ODN is also a more potent adjuvant (at least 4-fold under the experimental conditions used) than free ISS-ODN for cellular responses in this model system.

Based on the IgG2a/IgG1 ratio (≥5, Table 10) and the induction of cellular responses (Table 11), lip ISS-ODN triggers a much stronger Th1-dominant response than free ISS-ODN.

In an additional experiment the results of which are shown in the following Table 12, a comparison was made between free/lip ISS-ODN (1018), free/lip M-ODN (1019) and free/lip recombinant human IL-2 (previously used by us as an adjuvant in this model) as adjuvants for the A/New Caledonia subunit vaccine. In general, mice were immunized i.m. as detailed above and pooled sera were tested by ELISA 3 and 8 weeks post-vaccination. Antigen-specific IgG titers were calculated as detailed in connection with Table 3. Empty liposomes alone were not tested.

Table 12 - Antigen-specific serum isotypes following intramuscular vaccination of BALB/c mice with a monovalent influenza subunit vaccine, alone and combined with free/liposomal ISS-ODN or M-ODN

Vaccine (n=5)	**IgG1 titer**		**IgG2a titer**		**IgG2a/IgG1 ratio**	
	3W	8W	3W	8W	3W	8W
1. HN alone 0.5 μg	200	350	<10	<10	<0.05	<0.03
2. HN + ISS-ODN 10 μg	180	300	100	250	0.56	0.83
3. HN + lip ISS-ODN 10 μg	150	400	450	500	3.0	1.25
4. HN + M-ODN 10 μg	250	800	<10	50	<0.04	0.06
5. HN + lip M-ODN 10 μg	750	2000	<10	80	<0.013	0.04
6. HN + IL-2 10 μg	300	500	<10	35	<0.03	0.07
7. HN + lip IL-2 10 μg	750	6000	150	300	0.2	0.05

Both free and lip ISS-ODN markedly enhanced only the IgG2 level, the latter being 2-4.5 times more potent. In contrast, M-ODN, particularly lip M-ODN, enhanced mainly the IgG1 response (3-6-fold vs. antigen alone). Lip IL-2 increased both isotypes, particularly IgG1. This experiment demonstrates that the type of adjuvant markedly affects the type of response: lip ISS-ODN (Th1), lip M-ODN (Th2) and lip IL-2 (Th1+Th2).

In conclusion, this series of experiments indicated the superior adjuvant activity of liposomal ISS-ODN over free ISS-ODN for influenza vaccines administered i.m. or

i.n. The stronger humoral and cellular responses elicited by Lip ISS-ODN correlated well with the greater protection against viral infection.

Furthermore, no toxicity (no change in body weight, breathing, mobility, fur) was apparent in mice immunized with Lip ISS-ODN once or twice, i.n. and i.m. No swelling or ulceration was noticed at the i.m. injection site.

Example 2 - *Enhancement of the systemic anti-viral humoral response and cellular response by liposomal ISS-ODN 1018 (Lip ISS-ODN) co-administered with Hepatitis B vaccine*

In addition to the influenza vaccines, the adjuvant activity of free and liposomal (lip) ISS-ODN 1018 with a recombinant (yeast-derived) hepatitis B surface antigen (HBsAg) particles, which serves as standard hepatitis B vaccine in humans, was tested in mice. In this experiment, BALB/c mice (females, 5-weeks old) were vaccinated i.m. with 0.2 μg (0.03 ml) of recombinant HBsAg (Rhein Biotech, Dusseldorf, Germany) on days 0 and 21, using naked antigen (the HBsAg particles alone) or "alum"-adsorbed antigen, (alhydrogel 2%, aluminum hydroxide gel adjuvant, Superfros Biosectior, Frederikssund, Denmark, used as the human vaccine, with an alum/antigen w/w ratio of 25:1), each alone and combined with free/lip ISS-ODN (10 μg, DMPC/DMPG 9:1 mole ratio, lipid:ODN w/w ratio of 100:1). As controls, the antigen was co-administered with lip M-ODN (lacking the CpG motif) or empty liposomes.

For assessment of the humoral response (Table 13) (antigen-specific IgG1 and IgG2a antibodies, and HBsAg neutralizing antibodies) animals were bled on days 21 (for primary response) and 35 (for secondary response). Antigen-specific IgG isotypes were tested by ELISA, starting at a 1/10 serum dilution (as described in connection with the influenza model detailed above). The reciprocal of the highest serum dilution yielding OD = 0.2 (after subtracting OD with antigen + normal serum) was considered the IgG1 and IgG2a titer. Specific, neutralizing anti-HBs antibodies were measured using a commercial microparticle enzyme immunoassay (IMx AUSAB, Abbott Laboratories, USA) on serum samples diluted 1/2-1/10.

The cellular response (proliferation, cytokine production, cytotoxicity) was tested on day 56 using pooled spenocytes of each group. The results are presented in the following Table 14. For IFNγ and IL-4 production, cells (5 x 10^6/well) were incubated in duplicate in 24-well plats in a final volume of 1 ml of complete DMEM medium + 5% FCS, alone and together 5 μg of HBsAg. Supernatants were collected after 48 h and IFNγ and IL-4 were tested by ELISA, using the Opt EIA Set (Pharmingen, USA). In parentheses, cytokine levels in non-stimulated cultures (incubated without antigen). For determining the specific cytotoxicity cells were incubated as above, using complete DMEM medium + 10% FCS + 5x10^{-5}M 2-mercaptoethanol and cytotoxicity was measured on day 6 (4 h ^{51}Cr release assay, 4000 target cells/well, in triplicate) against labeled non-transfected P815 and HBsAg-transfected P815 (generously provided by Dr. Joerg Reimann, University of Ulm, Germany), at effector/target (E/T) cell ratios of 50/1 and 5/1. Specific cytotoxicity was calculated after subtracting cytotoxicity against non-transfected P815. No significant specific cytotoxicity was obtained in spleen cells cultured without the antigen.

For determination of proliferation, cultures were carried out, in triplicate, in U-shaped 96-well plates, using 5x10^5 cells/well in a final volume of 0.2 ml of complete DMEM medium + 5% FCS+5x10^{-5}M 2-mercaptoethanol. Cells were incubated alone and together with HBsAg 4 μg/well (c) or 1 μg/well (d) for 96 h and then pulsed with 1 μCi ^{3}H-tymidine overnight.

Table 13 - Humoral response of BALB/c mice immunized i.m. with HBsAg or alum-adsorbed HBsAg, alone and combined with free/liposomal ISS-ODN

Vaccine (n=5)	IgG1 titer		IgG2a titer		IgG2a/IgG1 ratio		Anti-HBs (mIU/ml)	
	21 d	35 d	21 d	35 d	21 d	35 d	21 d	35 d
1. None	<10	<10	<10	<10	-	-	<10	<10
2. HBsAg 0.2 μg alone	100	1,500	<10	350	<0.1	0.23	15	823
3. HBsAg + ISS-ODN 10 μg	60	4,000	15	2500	0.25	0.62	18	1,183
4. HBsAg + lip ISS-ODN 10 μg	450	50,000	150	20,000	0.33	0.4	235	6,402
5. HBsAg + lip M-ODN 10 μg	500	20,000	<10	650	<0.02	0.03	25	2,286
6. HBsAg + empty liposomes	550	10,000	<10	250	<0.02	0.02	90	2,868
7. Lip ISS-ODN 10 μg alone	<10	<10	<10	<10	-	-	<10	<10
8. Alum-HBsAg alone	300	6,500	<10	30	<0.03	0.005	61	3,510
9. Alum-HBsAg + ISS-ODN	80	6,500	45	7,000	0.56	1.08	89	4,653
10. Alum-HBsAg + lip ISS-ODN	300	25,000	300	35,000	1.0	1.4	99	8,693

Table 14-The cellular response of BALB/c mice immunized i.m. with HBsAg or alum-adsorbed HBsAg, alone and combined with free/liposomal ISS-ODN

Vaccine	IFNγ (pg/ml) [a]	IL-4 (pg/ml)[a]	% Specific cytotoxicity E/T=50/1	% Specific cytotoxicity E/T=5/1	Proliferation (SI) [b]
1. None	34 (0)	0 (0)	0	0	1.0[c]/1.0[d]
2. HBsAg 0.2 μg alone	60 (0)	0 (0)	0	0	2.2/1.1
3. HBsAg + ISS-ODN 10 μg	260 (0)	0 (0)	14	2	1.7/1.1
4. HBsAg + lip ISS-ODN 10 μg	1300 (0)	15 (0)	35	11	1.8/1.4
5. HBsAg + lip M-ODN 10 μg	11 (0)	28 (0)	0	0	2.5/1.9
6. HBsAg + empty liposomes	62 (0)	0 (0)	2	0	2.2/1.7
7. Lip ISS-ODN 10 μg alone	11 (0)	0 (0)	0	0	1.1/1.1
8. Alum- HBsAg alone	180 (0)	20 (0)	5	0	3.5/2.6
9. Alum- HBsAg + ISS-ODN	420 (0)	12 (0)	24	3	2.0/1.9
10. Alum- HBsAg + lip ISS-ODN	1400 (0)	16 (0)	46	14	3.3/3.0

[a] 0 = <10 pg/ml of either cytokine.

[b] Stimulation index (SI) = mean cpm cells + antigen/mean cpm cells w/o antigen ([c] 4μg/well, [d] 1μg/well).

As can be seen in Table 13, free ISS-ODN administered together with HBsAg (group 3) was ineffective for the primary response (day 21) and moderately effective for the secondary response (day 35), increasing the levels of antigen-specific IgG1, IgG2a and anti-HBsAg antibodies 2.7, 7.4 and 1.4-fold, respectively, compared with antigen alone (group 2). In comparison, lip-ISS-ODN (group 4) was very effective for both the primary and the secondary response, being 5.4-13 times more efficient than free ISS-ODN for the various antibodies tested at the two time points. Interestingly, the combination of antigen and empty liposomes or lip M-ODN (groups 5, 6) induced high levels of IgG1 (but not IgG2a) and anti-HBs antibodies directing the response to a Th2-type (IgG2a/IgG1 ratio $\leq$ 0.03). Thus, the elevation of IgG1 by lip M-ODN can partly be attributed to the stimulatory action of the liposomes themselves. In contrast, free ISS-ODN and lip ISS-ODN elicted a mixed Th1-Th2 response (ratio, 0.25-0.62).

Vaccination with alum-adsorbed antigen (group 8) induced, as expected, high levels of both IgG1 and anti-HBs antibodies and a strong Th2 response (IgG2a/IgG1 ratio <0.03). When combined with free or lip ISS-ODN (groups 9, 10), again a mixed Th1-Th2 response (ratio, 0.56-1.4) was noted. Liposomal ISS-ODN was also 2-7 more effective than free ISS-ODN in combination with alum-HBsAg for the various antibodies tested.

Collectively, these results are in accordance with the data obtained in the influenza model described above (Tables 2–12), demonstrating the superiority of lip ISS-ODN over free ISS-ODN for enhancing the humoral response. However, whereas a Th1-dominant response was induced by lip ISS-ODN in the influenza model, when injected i.m., a mixed Th1-Th2 response is generated in the hepatitis B model.

The cellular responses were tested at 8 weeks, 5 weeks after the second vaccine dose (Table 14). Splenocytes were incubated alone and together with the antigen, and IFNγ, IL-4, cytotoxicity and proliferative response were measured. For the cytotoxic response, cultures were also carried out with 30 IU/ml of recombinant human IL-2 (Chiron, USA). IFNγ was produced only in antigen-stimulated cultures. Vaccination with HBsAg administered together with free ISS-ODN increased IFNγ production 4.7-

fold compared with antigen alone; lip ISS-ODN induced 5 and 22 times more IFNγ than free ISS-ODN (groups 3, 4) and antigen alone (group 2), respectively. Lip M-ODN reduced IFNγ production and empty liposomes had no effect. Higher levels of IFNγ were generated following vaccination with alum-HBsAg, with and without free/lip ISS-ODN (groups 8-10), than with HBsAg without alum. Lip ISS-ODN was 3.3 times more efficacious than free ISS-ODN. IL-4 levels were low (0–28 pg/ml) in all the vaccinated mice.

Significant specific cytotoxicity against HBsAg transfected P815 target cells was observed only in the groups immunized with HBsAg + free/lip ISS-ODN (groups 3, 4, 9, 10). The cytotoxicity obtained with lip ISS-ODN was 2-5-fold (depending on the formulation and the effector/target cell ratio) higher than that induced with free ISS-ODN. Cultures containing IL-2 showed a slightly higher cytotoxic activity with a similar pattern. In contrast with the stimulatory effects of free/lip ISS-ODN on IFNγ production and cytotoxicity, there was no effect of either formulation on the proliferative response, which was low (stimulation index, 1.1-3.5) in all the groups. It is possible that the high level of IFNγ produced in the cultures blocked responder cell proliferation, or that the assay conditions were not optimal.

In conclusion, Table 14 shows that lip ISS-ODN was up to 5 times more potent than free ISS-ODN as an adjuvant with HBsAg vaccine with regard to IFNγ production and cytotoxic activity. These finding complement the humoral response data (Table 13), indicating that lip ISS-ODN is a powerful Th1-dominant adjuvant at a low dose (10 μg). In view of the ability of lip ISS-ODN to markedly boost both humoral and cellular responses to HBsAg, a combined vaccine consisting of the currently used HBV vaccine (alum- HBsAg) and lip ISS-ODN may be valuable not only as a prophylactic vaccine, but as a therapeutic vaccine in chronic HBV carriers with active hepatitis B as well.

Example 3 ***Enhancement of the systemic humoral response by liposomal ISS-ODN (Lip ISS-ODN) administered together with Tuberculosis vaccines***

In a preliminary experiment, lip ISS-ODN 1018 was tested in mice as an adjuvant for a new recombinant *M. tuberculosis* vaccine. A combined vaccine composed of *M. tuberculosis* recombinant proteins (ESAT-6, L7/L12 and 85 B) mixed with Ribi adjuvant (Sigma, USA) was administered s.c. twice to female BALB/c mice (6-weeks old), alone and together with lip ISS-ODN (10 μg/dose, prepared as described above). Free ISS-ODN was not tested in this experiment. Antigen-specific IgG1 and IgG2a were quantified by ELISA 3 weeks after the second vaccine dose. The results are shown in the following Table 15.

Table 15-Serum IgG isotypes of BALB/c mice immunized s.c. with recombinant *M. tuberculosis* proteins, with and without lip ISS-ODN

Vaccine (n=3)	**Mean optical density with:** [a]					
	ESAT-6		**L7/L12**		**85B**	
	IgG1	**IgG2a**	**IgG1**	**IgG2a**	**IgG1**	**IgG2a**
Ag + Ribi	0.178	0.104	0.285	0.116	0.210	0.190
	(0.6)		(0.4)		(0.9)	
Ag + Ribi + lip ISS-ODN	0.363	0.498	0.411	0.660	0.358	0.633
	(1.4)		(1.6)		(1.8)	
Fold increase	2.0	4.8	1.4	5.7	1.7	3.3

[a] Tested by ELISA against each antigen individually on sera diluted 1/4000; background OD reading was 0.05 (in parentheses, the IgG2a/IgG1 ratio)

As shown in Table 15, administration of lip ISS-ODN together with the tuberculosis vaccine increased both IgG1 and IgG2a against the 3 proteins, particularly IgG2a (1.4-2-fold vs. 3.3-5.7-fold, respectively, for IgG1 and IgG2a). This resulted in an increased IgG2a/IgG1 ratio from 0.6, 0.4 and 0.9 without lip ISS-ODN to 1.4, 1.6 and 1.8 with lip ISS-ODN, respectively, indicating a shift to a Th1 response.

In an additional experiment, Lip ISS-ODN 1018 was compared with free ISS-ODN as an adjuvant for Tuberculosis vaccine comprising a different mixture of antigens. According to this specific experiment, In general, BALB/c mice (females, 2-months-old, n=5/group) were vaccinated subcutaneously on days 0 and 21 using a mixture of 3 different antigens (5 μg each) derived from *M. tuberculosis*. ODN 1018-10 μg/dose. The results presented in Table 16 show that lip ISS-ODN was 2–2.5-fold more potent for two of the three antigens tested.

These results suggest that lip ISS-ODN may prove beneficial as a parenteral Th1 adjuvant for future tuberculosis vaccines.

Table 16-Soluble and liposomal ISS-ODN (ODN 1018) as adjuvants for tuberculosis vaccine in mice (exp. 2)

Vaccine	**Antigen**	**IgG1 titer ($x10^3$)[a]**	**IgG2a titer ($x10^3$)**	**IgG2a/IgG1 ratio**
Antigen alone	Sod	13	7	0.54
Antigen + ODN 1018		12	11	0.91
Antigen + lip ODN 1018		**22**	**26**	**1.18**
Antigen alone	85B	11	8	0.72
Antigen + ODN 1018		10.5	8	0.76
Antigen + lip ODN 1018		13	**16.5**	**1.27**
Antigen alone	CFP21	21	8.5	0.4
Antigen + ODN 1018		110	23	0.21
Antigen + lip ODN 1018		105	22	0.21

[a] Pooled sera were tested by ELISA 3 weeks after the second vaccine dose. ELISA titer was determined as the highest serum dilution yielding OD=0.2.

Example 4 ***Lip ISS-ODN 1018 as an adjuvant for cancer vaccine***

A weakly immunogenic murine mammary carcinoma (*4T1*) was used in 2 experiments. Female, 2-months-old (BALB/c x C57BL6) F1 mice were vaccinated 3 times, intradermally, on days 0, 10 and 20 with either irradiated tumor cells only, tumor cells admixed with 10 μg per dose of free or liposomal ISS-ODN 1018, or with empty liposomes. Tumor challenge was injected subcutaneously 10 days after the last vaccination (injection of $5x10^4$ live tumor cells) and mice were inspected for tumor incidence and size 4 weeks later. As shown in Table 17, only groups co-vaccinated with ISS-ODN were partially protected, with 33% and 25% protection achieved with Lip ISS-ODN and free ISS-ODN, respectively. In a similar experiment the protection rate was 37% (3/8) vs. 25% (2/8), respectively. Thus, under the experimental conditions used, Lip ISS-ODN was slightly more effective than free ISS-ODN. Optimization of the vaccination procedure is required to further increase the protection rate.

Table 17-Soluble and liposomal ISS-ODN (ODN 1018) as adjuvants for cancer vaccine in mice

Vaccine[a]	**No. of tumor-bearing mice/total**[b] **on day 28**	**Mean tumor size (cm^3)**[b] **on day 28**
1. None	8/8	1.7
2. Tumor alone	8/8	0.6
3. Tumor + soluble ODN 1018	6/8	0.44
4. Tumor + liposomal ODN 1018	**6/9**	**0.29**
5. Tumor + empty liposomes	8/8	0.78

[a] vaccination with 10^7 irradiated (20,000R) 4T1 cells at 4 sites either alone or together with soluble or liposomal ODN 1018 or with empty liposomes as described.

[b] Live tumor cells ($5x10^4$) were injected intradermally 10 days after the third vaccine dose.

Example 5 ***Activation of resistance to Leishmaniasis by liposomal ISS-ODN (Lip ISS-ODN) administered after infection***

Resistance to leishmaniasis requires activation of a Th1-type response. Therefore, free and liposomal (lip) ISS-ODN 1018 were tested as post-infection treatment for leishmaniasis in BALB/c mice. In particular, animals (8-9/group) were injected subcutaneously (s.c.) in the tail base with 250,000 Leishmania major promastigotes. Saline, free ISS-ODN, lip ISS-ODN or lip M-ODN (lacking the CpG motif) were administered intramuscularily (i.m., 0.1 ml) at 20 μg/dose on days 1, 7, 14 (cycle 1) and 72 and 82 (cycle 2) post infection (lip ISS-ODN or lip M-ODN comprising DMPC/DMPG at mole ratio of 9:1 and lipid:ODN ratio (w/w) of 100:1, prepared as described above). Subcutaneous lesions were measured weekly by caliper.

As shown in Table 18 and in Fig. 1, free ISS-ODN demonstrated a moderate protective effect. Animals treated with lip ISS-ODN showed a slower tempo of disease development, and the lesion size after each treatment cycle was about half of that seen in the other groups, including mice treated with free ISS-ODN.

In two additional experiments mice were infected as above, then treated with a subcurative dose of anti-leishmanial drugs (Amphotericin B (Fig. 2) and Amphotericin B derivative (Fig. 3)), alone and combined with lip ISS-ODN. Other groups were treated with either lip ISS-ODN or lip M-ODN (lacking the CpG motif) only. As shown in Figs. 2 and 3, a synergistic therapeutic effect was achieved in the group co-treated with the drug and the lip ISS-ODN. Since the therapeutic effect is transient and partial, repeated treatment over a long period of time, or a combination of higher doses of lip ISS-ODN with higher doses of anti-leishmanial drugs may be necessary to achieve a complete cure.

Table 18-Post-infection treatment of leishmaniasis in BALB/c mice with free/liposomal ISS-ODN (cycle 1)

Treatment[a]	**Lesion size (mm^2, mean ± SD) on week:** [b]	
	4	**8**
Saline	1.44 ± 1.22 (1/8)	11.67 ± 7.9 (0/8)
ISS-ODN 20 μg	0.74 ± 1.08 (4/9)	8.3 ± 6.8 (1/9)
Liposomal ISS-ODN 20 μg	0.46 ± 1.04 (6/9)	3.8 ± 3.5 (1/9)
Liposomal M-ODN 20 μg	1.35 ± 1.67 (2/8)	13.2 ± 10.1 (1/8)

[b] Two diameters were measured for calculation of the lesion area. In parentheses, number of mice without lesions/total number of mice.

CLAIMS:

1. A method for loading immunostimulatory oligodeoxynucleotides (ISS-ODNs) in liposomes comprising:

(a) solubilizing at least one liposome-forming lipid in a solvent and drying same to effect a dry liposome-forming lipid or dry mixture of lipids;

(b) providing an aqueous solution of ISS-ODN;

(c) hydrating the dry liposome-forming lipid or mixture of lipids with said ISS-ODN solution to achieve loading of said ISS-ODN in liposomes.

2. A method for loading immunostimulatory oligodeoxynucleotides (ISS-ODNs) in liposomes comprising:

(a) solubilizing at least one liposome-forming lipid in a solvent and freeze-drying same to effect a dry lipid or dry mixture of lipids;

(b) providing an aqueous solution of ISS-ODN;

(c) hydrating the freeze-dry lipid or mixture of lipids with said ISS-ODN solution to achieve loading of said ISS-ODN in liposomes.

3. The method of Claim 1 or 2, wherein said liposome-forming lipid comprises phospholipid, lipopolymers, sphingolipids a combination thereof and a combination thereof with sterols.

4. The method of Claim 3, wherein said phospholipid is selected from saturated or unsaturated phospholipids, hydrogenated, partially hydrogenated or non-hydrogenated phospholipids, fully or semi synthetic phospholipids.

5. The method of Claim 3, wherein said hydrogenated, partially hydrogenated or non-hydrogenated phospholipids are derived of natural sources, said natural source is selected from egg yolk, milk, rice or soybeans.

6. The method of Claim 4, wherein said fully synthetic or semi-synthetic phospholipids are selected from dimyristoyl phosphatidylcholine (DMPC), dimyristoyl

phosphatidylglycerols (DMPG), phosphatidylglycerols, phosphatidylinositols, phosphatidylserines, sphingomyelins, or mixture thereof.

7. The method of Claim 6, wherein the liposome-forming lipids comprises a mixture of DMPC and DMPG.

8. The method of Claim 7, wherein said mixture of DMPC and DMPG is at a molar ratio of between 1:20 and 20:1

9. The method of Claim 3, wherein said lipopolymer is a PEGylated lipid.

10. The method of Claim 3, wherein said sphingolipids are sphingomyelins (SPM) selected from egg-derived SPM, milk-derived SPM, N-palmitoyl-SPM, N-stearoyl-SPM, N-oleoyl-SPM (C18:1), N-nervacyl C (C24:1) SPM, N-lignoceryl SPM (C24:0), or a mixture thereof.

11. The method of Claim 1 or 2, wherein said ISS-ODN is loaded into said liposome in combination with an antigen or mixture of antigens.

12. The Method of Claim 1 or 2, wherein said ISS-ODN is an endotoxin-free ISS-ODN with a phosphorothioate (PS) or phosphodiester (PO) backbone.

13. The method of Claim 1 or 2, wherein said solvent is a polar, water miscible solvent.

14. The method of Claim 13, wherein said solvent is tertiary-butanol.

15. The method of Claim 1 and 2 wherein the solvent is an apolar solvent.

16. The method of Claim 15, wherein said apolar solvent is cyclohexane.

17. The method of Claim 1, wherein said drying includes spray drying.

18. The method of Claim 1 or 2, wherein said solution of ISS-ODN is a solution thereof in sterile pure water, a physiologically acceptable aqueous solution selected from 0.9% NaCl, buffered Saline, 5% dextrose, buffered dextrose, 10% sucrose and buffered sucrose or cryprotectant or a mixture of same.

19. A combination of two pharmaceutical compositions including a first pharmaceutical composition comprising dry liposome-forming lipids and a second pharmaceutical composition comprising ISS-ODN, the combination is for use in the preparation of a pharmaceutical formulation comprising liposomal ISS-ODN.

20. The combination of Claim 19, being in the form of a package.

21. The combination of Claim 19 or 20, comprising instructions for use of said first pharmaceutical composition and of said second pharmaceutical composition for the preparation of said pharmaceutical composition, the instructions comprising hydrating the dry liposome forming lipid or mixture of lipids of the first composition with an aqueous solution of the second composition containing ISS-ODN; and further comprising instructions prescribing administration of said pharmaceutical formulation to a subject in need thereof.

22. The combination of Claim 21, comprising a physiologically acceptable aqueous medium, sterile water and/or cryprotectant, for forming a solution of ISS-ODN.

23. The combination of Claim 19, wherein the liposome-forming lipid comprises phospholipid, lipopolymers, sphingolipids and combination of the same with sterols

24. The combination of Claim 23, wherein said liposome-forming lipid comprises saturated or unsaturated phospholipids, hydrogenated, partially hydrogenated or non-hydrogenated phospholipids, fully or semi synthetic phospholipids.

25. The combination of Claim 24, wherein said hydrogenated, partially hydrogenated, or non-hydrogenated phospholipids are derived from a natural source; said natural source is selected from egg yolk, milk, rice or soybeans.

26. The combination of Claim 24, wherein said fully synthetic or partially synthetic phospholipids are selected from dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG), phosphatidylglycerol, phosphatidyl–inositol, phosphatidylserine, or mixture thereof.

27. The combination of Claim 26, wherein said liposome forming lipid comprises a mixture of DMPC and DMPG.

28. The combination of Claim 27, wherein said mixture DMPC and DMPG is at a molar ratio of between 1:20 and 20:1.

29. The combination of Claim 23, wherein said lipopolymer is PEGylated lipids.

30. The combination of Claim 23, wherein said sphingolipids are sphingomyelins (SPM) selected from egg-derived SPM, milk-derived SPM, N-palmitoyl-SPM, N-stearoyl-SPM, N-oleoyl-SPM (C18:1), nervonyl C (C24:1) SPM, N-lignoceryl SPM (C24:0), or a mixture thereof.

31. The combination of Claim 19, wherein said ISS-ODN is an endotoxin-free ISS-ODN with a phosphorothioate (PS) or phosphodiester (PO) backbone.

32. The combination of Claim 19 or 31, wherein said second composition comprises, in combination with said ISS-ODN, an antigen or a mixture of antigens.

33. The combination of Claim 22 or 31, wherein said physiologically acceptable aqueous medium is sterile water or a solution selected from the group consisting of 0.9% NaCl, buffered Saline, 5% dextrose, buffered dextrose, 10% sucrose, buffered sucrose and mixtures of the same.

34. A pharmaceutical formulation comprising as active ingredient a therapeutically effective amount of liposomes loaded with an ISS-ODN and a pharmaceutically acceptable additive, the liposomal ISS-ODN being prepared by the method of any one of Claims 1 to 17.

35. The pharmaceutical composition of Claim 34, wherein said effective amount is a dosage of up to 2,000 mg of loaded liposomal vesicles, measured by phospholipid, per kg body weight.

36. The pharmaceutical formulation of Claim 34 comprising, in combination with said ISS-ODN, an antigen or a mixture of antigens, said antigen(s) is in a free form or loaded into a liposome.

37. The pharmaceutical formulation of Claim 36, wherein said antigen is loaded together with the ISS-ODN in the same liposome or in separate liposomes.

38. The pharmaceutical formulation of any one of Claims 34 to 37, for eliciting an immune response of an individual.

39. A method for the prevention or treatment of a disease comprising administering to a subject in need an effective amount of pharmaceutical formulation according to any one of Claims 34 to 38.

40. A method for stimulating the immune response of an individual, the method comprising administration to said individual an amount of ISS-ODN-loaded liposome effective to stimulate said immune response, wherein said stimulation is to an extent greater than that obtained by administration to the individual free ISS-ODN, the ISS-ODN-loaded liposome being prepared by the method of any one of Claims 1 to 19.

41. The method of Claim 40, wherein said amount is a dosage of up to 2,000 mg of loaded liposomal vesicles, measured by phospholipid, per kg body weight.

42. The method of Claim 40, comprising administration of said liposomal ISS-ODN in combination with at least one antigen.

43. The method of Claim 42, wherein said antigen is in a free form.

44. The method of Claim 42, wherein said antigen is encapsulated together with said ISS-ODN in the same liposomes.

45. The method of Claim 42, wherein said antigen is encapsulated in a liposome separate from said ISS-ODN.

Fig. 1

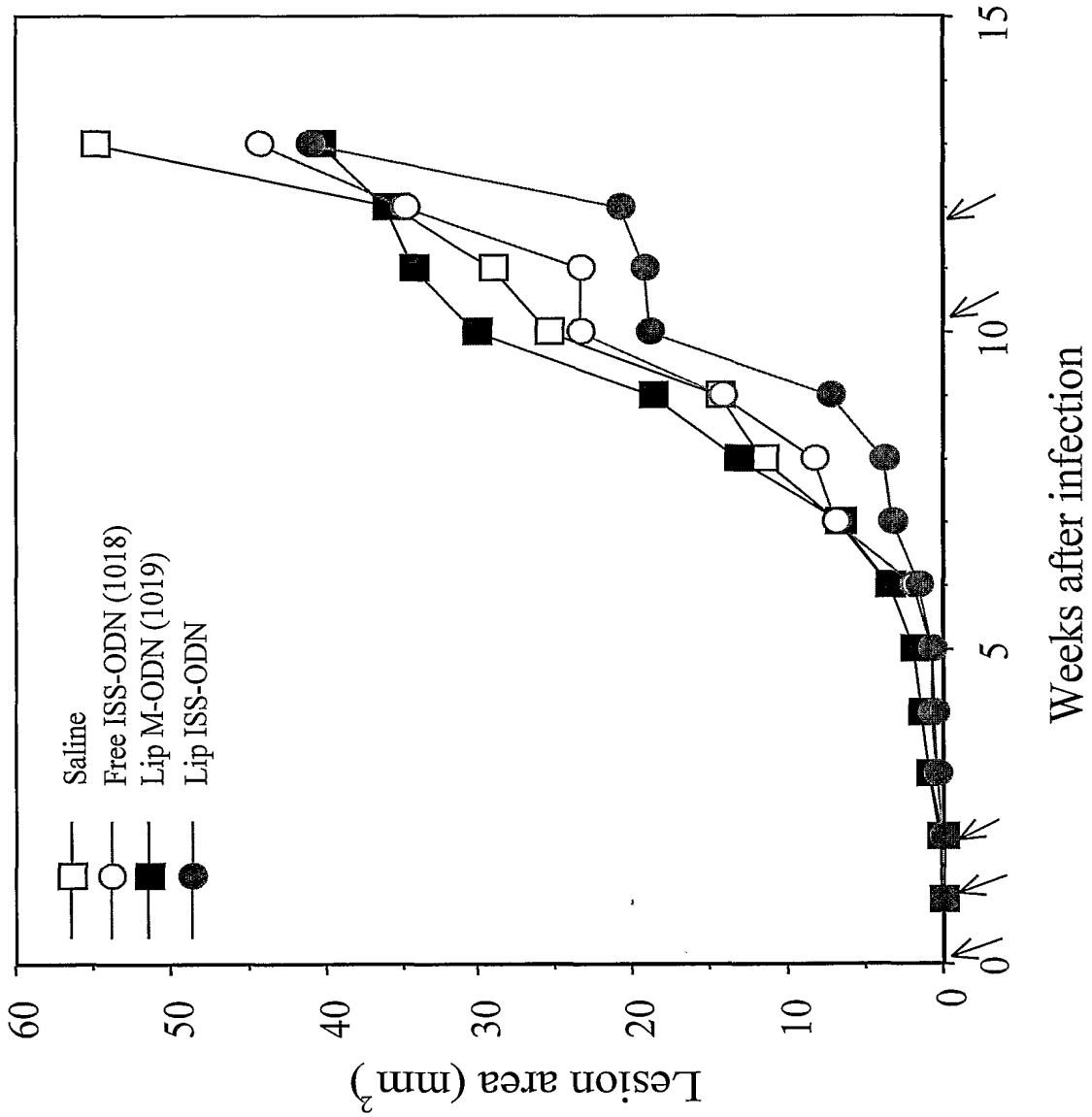

Lesion area (mm²)
60
50
40
30
20
10
0
0
5
10
15
Weeks after infection
Saline
Free ISS-ODN (1018)
Lip M-ODN (1019)
Lip ISS-ODN

Fig. 2

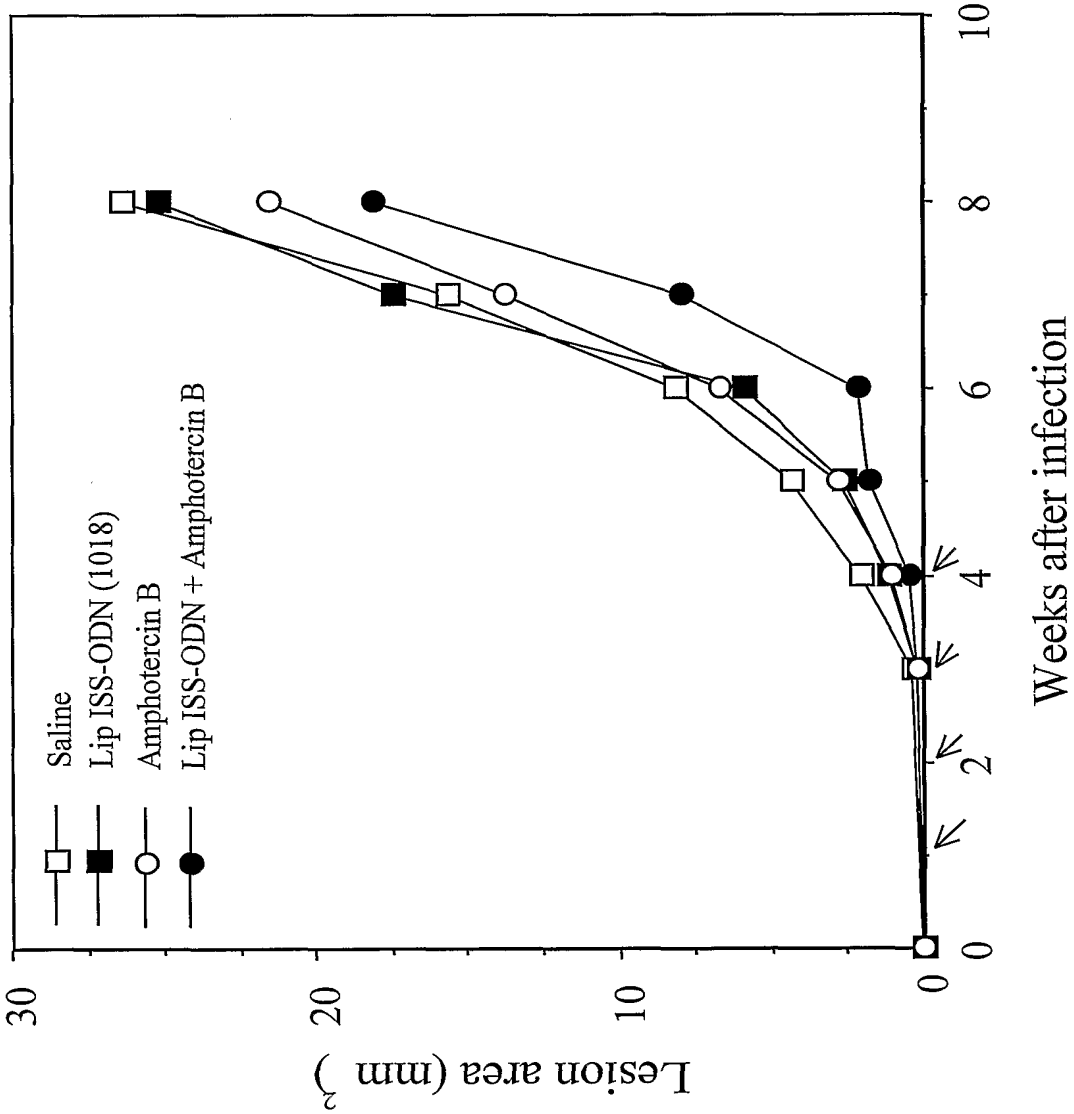
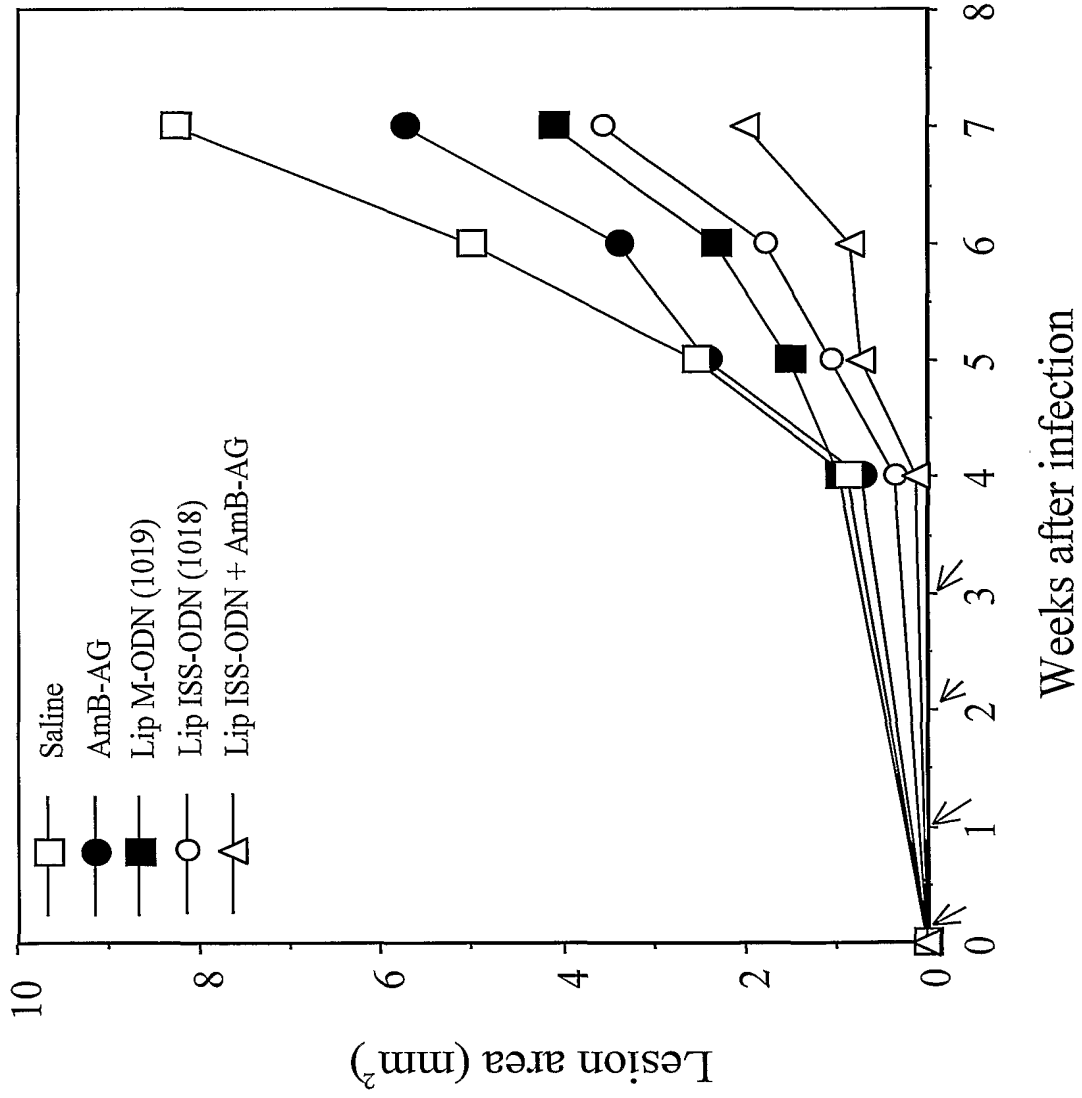

Lesion area (mm 2)
30
20
10
0
0
2
4
6
8
10
Weeks after infection
Saline
Lip ISS-ODN (1018)
Amphotercin B
Lip ISS-ODN + Amphotercin B

Fig. 3


Lesion area (mm²)
10
8
6
4
2
0
0
1
2
3
4
5
6
7
8
Weeks after infection
Saline
AmB-AG
Lip M-ODN (1019)
Lip ISS-ODN (1018)
Lip ISS-ODN + AmB-AG

SEQUENCE LISTING

<110> YISSUM RESEARCH DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM

<120> A METHOD FOR PREPARATION OF VESICLES LOADED WITH IMMUNOSTIMULATORY OLIGODEOXYNUCLEOTIDES AND DIFFERENT USES THEREOF

<130> 1378793

<150> US 60/300,072
<151> 2001-06-25

<150> US 60/339,785
<151> 2001-12-17

<160> 4

<170> PatentIn version 3.1

<210> 1
<211> 22
<212> DNA
<213> Synthetic

<400> 1
tgactgtgaa cgttcgagat ga
22

<210> 2
<211> 22
<212> DNA
<213> Synthetic

<400> 2
tgactgtgaa ggttagagat ga
22

<210> 3
<211> 23
<212> DNA
<213> Synthetic

<400> 3
tccataacgt tgcaaacgtt ctg
23

<210> 4
<211> 23

<212> DNA
<213> Synthetic

<400> 4
tccatgacgt tcctgacgtt ctg
23